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# ATP-Sensitive $K^+$ Channels in Insulinoma Cells Are Activated by Nonesterified Fatty Acids†

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ABSTRACT: Both $^{86}Rb^+$ efflux experiments and electrophysiological studies have shown that arachidonic acid and other nonesterified fatty acids activate ATP-sensitive $K^+$ channels in insulinoma cells (HIT-T15). Activation was observed with arachidonic, oleic, linoleic, and docosahexaenoic acid but not with myristic, stearic, and elaidic acids. Fatty acid activation of ATP-sensitive $K^+$ channels was blocked by antidiabetic sulfonylureas such as glibenclamide. The activating effect of arachidonic acid was unaltered by indomethacin and by nordihydroguaiaretic acid, indicating that it is not due to metabolites of arachidonic acid via cyclooxygenase or lipoxygenase pathways. Moreover, the nonmetabolizable analogue of arachidonic acid, eicosatetraynoic acid, was an equally potent activator. Activation of ATP-sensitive $K^+$ channels by fatty acids was potentiated by diacylglycerol and was inhibited by calphostin C, an inhibitor of protein kinase C. These findings indicate that fatty acid activation of ATP-sensitive $K^+$ channels is most likely due to the participation of arachidonic acid (and other fatty acid)-activated protein kinase C isoenzymes. Activation of ATP-sensitive $K^+$ channels by nonesterified fatty acids is not involved in the control of insulin secretion since arachidonic acid stimulates insulin secretion from insulinoma cells instead of inhibiting it.

ATP-sensitive $K^+$ ($K_{ATP}$)[1] channels play a key role in insulin secretion from pancreatic $\beta$-cells (Ashcroft, 1988). Exposure of $\beta$-cells to glucose induces closure of $K_{ATP}$ channels via a variation of the intracellular ATP/ADP ratio and thereby a depolarization which leads to activation of $Ca^{2+}$ channels, elevation of intracellular $Ca^{2+}$, and insulin secretion (Dunne & Petersen, 1991). $K_{ATP}$ channels are specifically blocked by sulfonylureas such as glibenclamide which are drugs capable of restoring insulin secretion in patients affected by non-insulin-dependent diabetes (Schmid-Antomarchi et al., 1987). They are activated by hormones such as somatostatin and galanin which inhibit insulin secretion (De Weille et al., 1988, 1989; Dunne et al., 1989).

Arachidonic acid (AA) and its metabolites are known to be modulators of $K^+$ channels in several cell types. For example, 5-lipoxygenase-derived metabolites of AA (leukotriene $B_4$ and $C_4$) modulate the activity of muscarinic cardiac $K^+$ channels (Kim & Clapham, 1989; Kim et al., 1989; Kurachi et al., 1989), while AA derivatives from the 12-lipoxygenase pathway (12-HPETE) act directly on $K^+$ channels of the S-type in nervous cells from *Aplysia* (Belardetti et al., 1987, 1989; Piomelli et al., 1987). A class of $K^+$ channels from smooth muscle cells is activated directly by nonesterified fatty acids (NEFAs) (Ordaway et al., 1989).

## Experimental Procedures

*Materials.* Indomethacin, NDGA, 1,2-dioleoylglycerol (DOG), 1-oleoyl-2-acetylglycerol (OAG), oligomycin, penicillin, polyornithine, streptomycin, trypsin, and all NEFAs with the exception of ETYA (Fluka, Mulhouse, France) were from Sigma (L'Isle d'Abeau, France). Calphostin C was from Kamiya Biomedical Co. (Thousand Oaks, CA). Ham's F12k medium was from Gibco (Paisley, U.K.). Glibenclamide and tolbutamide were from Hoechst Laboratories (Paris, France);

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[1] Abbreviations: AA, arachidonic acid; DOG, 1,2-dioleoylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ETYA, eicosatetraynoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $K_{ATP}$ channels, ATP-sensitive $K^+$ channels; NEFAs, nonesterified fatty acids; NDGA, nordihydroguaiaretic acid; OAG, 1-oleoyl-2-acetylglycerol.

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glisoxepide was from Shering-Plough (Paris, France); horse and fetal calf sera were from Dutcher (Brumath, France). $^{86}RbCl$ was from Amersham (Les Ulis, France). All other reagents were of the highest available purity.

*Cell Culture.* HIT-T15 β-cells (ATCC, passage no. 59) were cultured in Ham's F12k medium supplemented with 10% dialyzed horse serum and 2.5% fetal calf serum (both inactivated), 100 units/mL penicillin, and 0.1 mg/mL streptomycin in humidified 5% $CO_2$/95% air at 37 °C. The culture medium was changed every 3–4 days. Culture flasks (Corning, 225 $cm^2$) were currently used with (14–16) × $10^6$ cells in 100 mL of medium. Cells were passaged weekly and harvested using trypsin–EDTA. Cells were seeded in 24-well plates (Falcon 3017) at a density of (3–5) × $10^5$ cells/mL per well, 2–4 days before $^{86}Rb^+$ efflux experiments, and the culture medium was supplemented with 0.2 μCi/mL [$^3$H]leucine. Twenty-four-well plates were treated with 50 μg/mL polyornithine and washed three times before use with a medium containing (in mM) NaCl (116), KCl (5.3), D-glucose (5), $NaH_2PO_4$ (8), and $NaHCO_3$ (22.6) as well as phenol red (10 mg/mL). Cells were used from passage no. 62 to 73.

*$^{86}Rb^+$ Efflux Experiments.* Radioactive rubidium efflux experiments were carried out essentially as previously described (Müller et al., 1991). Briefly, after the culture medium was removed, cells were preincubated at pH 7.5 in a medium containing (in mM) NaCl (120), KCl (10), $CaCl_2$ (1.8), $MgCl_2$ (0.8), and HEPES–NaOH (20), supplemented with 0.1 μCi/mol $^{86}RbCl$ (250 μL/well). After 120–150 min of incubation, efflux experiments were started by removing the medium containing $^{86}RbCl$ and replacing it with the same medium without $^{86}RbCl$. Preincubation with possible effectors of $K_{ATP}$ channels was performed in the plate wells in the presence of $^{86}RbCl$. Efflux was stopped at the indicated times (see figures) by washing the cells with 1 mL of the $^{86}RbCl$-free medium. Cells were then extracted with 1 mL of 0.1 M NaOH, mixed with Aquassure (NEN, Les Ulis, France) as scintillation fluid and counted for radioactivity. All experiments were carried out at 37 °C. Results of $^{86}Rb^+$ flux experiments were expressed as the percent of $^{86}Rb^+$ remaining in cells or as the percent of $^{86}Rb^+$ efflux (100% – % of $^{86}Rb^+$ remaining in cells).

*Electrophysiology.* Current-clamp experiments were carried out on HIT-T15 β-cells using the whole-cell suction-pipet technique (Hamill et al., 1981). Single-channel curents were recorded from inside-out and outside-out membrane patches with membrane potentials clamped at 0 mV by a voltage-clamp amplifier (Bio-Logic, Grenoble, France). Membrane potentials were recorded using the perforated patch technique (Horn & Marty, 1988). Nystatin (Gibco, Paisley, U.K.) was dissolved in dimethyl sulfoxide, and pipets were back-filled with a solution containing a final concentration of 500–5000 units/mL nystatin. In all experiments, the intracellular solution contained KCl (150 mM), $MgCl_2$ (1 mM), EGTA (2 mM), and HEPES–KOH (10 mM), pH 7.2. The extracellular solution was NaCl (140 mM), KCl (5 mM), $MgCl_2$ (2 mM), $CaCl_2$ (2 mM), and HEPES–NaOH (10 mM), pH 7.3. Pipets were coated with Sylgard resin to reduce current noise. Electrical signals were digitized by a digital oscilloscope (Nicolet, Madison, WI) and stored on hard disk by computer (Hewlett Packard, Sunnyvale, CA) for further analysis. Experiments were carried out at room temperature (~25 °C).

*Insulin Secretion.* Two or three days before each study, cells were subcultured at 4 × $10^6$ cells/2 mL per well in 6-well tissue culture plates (Falcon). The culture medium was replaced the day before measurements were started. On the day of insulin level measurements, cells were washed twice at 37 °C with 2 mL of Krebs–Ringer–bicarbonate–HEPES (KRB) buffer prepared as previously described (Wollheim et al., 1990). After washing, 2 mL of buffer was added and plates were incubated for 60 min at 37 °C. Cells were washed twice more prior to addition of 1 mL of KRB buffer containing the stated concentration(s) of test substance(s). After a 20-min incubation, supernatants were centrifuged (5 min, 500*g*) to remove cell debris and then stored at −20 °C until analyzed by radioimmunoassay. Supernatants were analyzed for insulin content within 3 days of collection. Cells were used from passage no. 62–66.

Immunoreactive insulin content in supernatants was determined by a radioimmunoassay using $^{125}I$-labeled pig insulin (Novo, Paris, France), guinea pig anti-porcine insulin antibody (Miles, Puteaux, France), and rat insulin (Novo, Paris, France) as a standard. All samples were diluted in sample buffer for the values to fall within the linear part of the standard curve and analyzed in triplicates.

## Results

Oligomycin-induced ATP depletion in insulinoma cells is known to cause the opening of $K_{ATP}$ channels and a sulfonylurea-sensitive $^{86}Rb^+$ efflux (Schmid-Antomarchi et al., 1987). This $^{86}Rb^+$ efflux technique was used to analyze the effect of NEFAs on this type of channel. Figure 1A shows that 20 μM AA produces a component of $^{86}Rb^+$ efflux that is completely inhibited by 1 μM glibenclamide, one of the most potent sulfonylureas (Schmid-Antomarchi et al., 1987). This result suggests that AA or one of its metabolites activates the $K_{ATP}$ channel. All metabolites are formed via the cyclooxygenase or the lipoxygenase pathways, which can be blocked by indomethacin and nordihydroguaiaretic acid, respectively. Activation of $K_{ATP}$ channel activity by AA was unaltered in the presence of indomethacin or nordihydroguaiaretic acid (Figure 1), suggesting that the activation is due to AA itself. A confirmation of this result was obtained by the observation that the nonmetabolizable analogue of AA, eicosatetraynoic acid (ETYA), produces the same effects as AA. Figure 1B shows the external concentration dependence of the ETYA opening effect. ETYA activation of $^{86}Rb^+$ efflux is inhibited by glibenclamide as expected for a sulfonyurea-sensitive $K_{ATP}$ channel.

Several antidiabetic sulfonylureas have been assayed for their inhibitory effect on ETYA activation of $K_{ATP}$ channels (Figure 2). The order of potency was glibenclamide ($IC_{50}$ = 0.5 nM) > glisoxepide ($IC_{50}$ = 63 nM) > tolbutamide ($IC_{50}$ = 50 μM). These $IC_{50}$ values for the different sulfonylureas are similar to those necessary to block $K_{ATP}$ channels after ATP depletion (Schmid-Antomarchi et al., 1987).

Electrophysiological results confirmed the observations made from $^{86}Rb^+$ efflux measurements (Figure 3). Application of AA or ETYA to HIT-T15 insulinoma cells leads to an hyperpolarization from −50 to −70 mV which is suppressed by the $K_{ATP}$ channel blocker glibenclamide. Again these results indicate that AA, and not one of its metabolites, activates sulfonylurea-sensitive $K_{ATP}$ channels. Panels A and B of Figure 3 show that membrane resistances do not return to control values after inhibition of $K_{ATP}$ channels with glibenclamide, indicating that another channel (possibly a $Cl^-$ or a nonselective cation conductance) is also activated by AA and ETYA. Due to the dual action of AA, the time course of membrane hyperpolarization probably does not exactly reflect the time course of $K_{ATP}$ channel activation.

AA and ETYA (Figure 3C,D) had no effect on $K_{ATP}$ channel activity in excised patch recordings excluding a direct

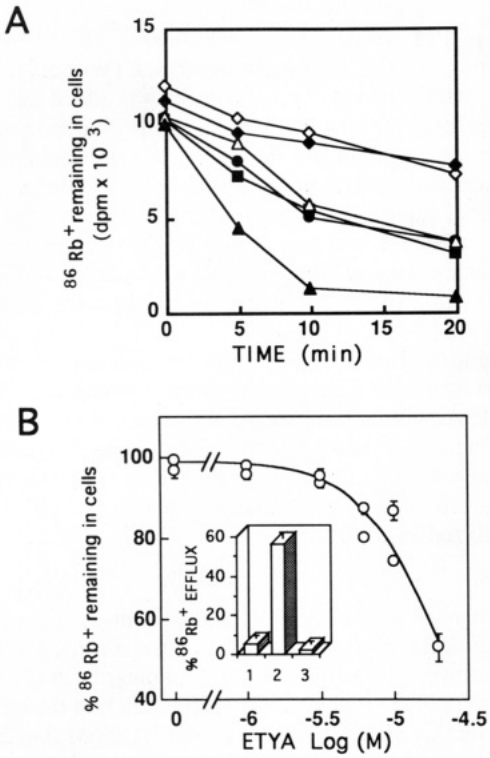


FIGURE 1: Nonesterified fatty acids open $^{86}Rb^+$-permeable sulfonylurea-sensitive $K^+$ channels in HIT $\beta$-cells. (A) Kinetics of $^{86}Rb^+$ efflux. Untreated control (◇). ATP depletion and subsequent $K_{ATP}$ channel opening was obtained by preincubating cells with 250 ng/mL oligomycin for 10 min prior to efflux measurement (▲). Preincubation with 20 $\mu$M arachidonic acid induced a $^{86}Rb^+$ efflux (■) which was sensitive to 1 $\mu$M glibenclamide (◆), but not to 9 $\mu$M indomethacin (●) nor to 3.3 $\mu$M nordihydroguaiaretic acid (△), both preincubated for 25 min. $^{86}Rb^+$ radioactivity was normed for [$^3$H]leucine incorporated in cells. Activation of $K_{ATP}$ channels by AA was observed in more than 130 experiments. (B) Concentration-dependent activation of $^{86}Rb^+$ efflux induced by eicosatetraynoic acid. $^{86}Rb^+$ efflux was performed for 30 min in the presence of the given ETYA concentrations without preincubation. Experimental data were normalized using untreated control as 100%. Activation of $K_{ATP}$ channels by ETYA was observed in more than 40 experiments. Inset: Column 1, untreated control; column 2, efflux produced by 20 $\mu$M ETYA over 30 min without preincubation; column 3, inhibition of ETYA induced efflux by 1 $\mu$M glibenclamide; $n = 2$.

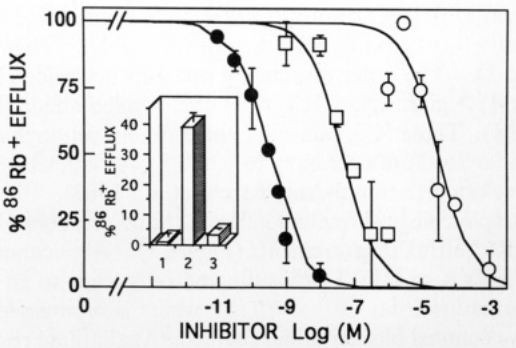


FIGURE 2: Inhibition by sulfonylureas of AA- and ETYA-activated $K^+$ channels. Concentration-dependent inhibition by sulfonylureas of the arachidonic acid-induced $^{86}Rb^+$ efflux in HIT $\beta$-cells. Cells were preincubated 10 min with 20 $\mu$M arachidonic acid. Flux was performed for 10 min in the presence of arachidonic acid and the corresonding conentration of sulfonylureas: glibenclamide (●), glisoxepide (□), and tolbutamide (○), $n = 2$. Inset: Column 1, untreated control; column 2, efflux produced by 20 $\mu$M arachidonic acid over 30 min without preincubation; column 3, inhibition of arachidonic acid induced efflux by 1 $\mu$M glibenclamide; $n = 2$.

effect of this type of fatty acid on this class of channel in insulinoma cells.

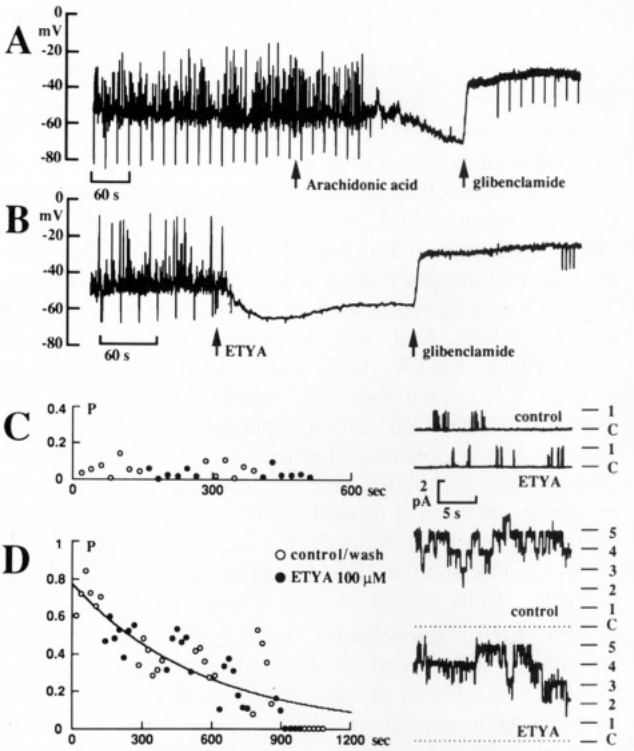


FIGURE 3: Effects of AA and ETYA on membrane potential in insulinoma cells. Upon extracellular application of AA (50 $\mu$M, A) or ETYA (30 $\mu$M, B), cells' hyperpolarized, electrical activity ceased and membrane resistance decreased. Subsequent application of the $K_{ATP}$ channel blocker, glibenclamide (100 nM), depolarized the membrane again, but electrical activity remained absent and membrane resistance remained low. Hyperpolarizing deflections in the traces were induced by 6-pA, 300-ms current pulses. The last few current pulses at the end of each trace were 60 pA. Outside-out membrane patches were excised with a pipet containing a $K^+$-rich solution and voltage-clamped at 0 mV. In the absence of nucleotides in the pipet solution, the probability of channel opening ($P$) was low and remained so after application of 100 $\mu$M ETYA to the patch (C). In the presence of 100 $\mu$M ADP at the intracellular face of the membrane, up to seven channels were seen to open simultaneously and their probability of opening was relatively high (D). The number of active channels gradually decreased during the experiment (shown in the diagram as a decrease of $P$). Neither channel rundown nor the activity of the individual channels was affected by 100 $\mu$M ETYA. The probability of opening shown in panels C and D was calculated from 20-s records filtered at 100 Hz. Examples of such records, each representing a single data point in panel C or D, are shown at the right-hand side.

Table I: Activation of $^{86}Rb^+$ Efflux in HIT-T15 $\beta$-Cells by Different Nonesterified Fatty Acids[a]

| fatty acid | concentration ($\mu$M) | % of $^{86}Rb^+$ efflux ± SD |
|---|---|---|
| untreated control | | 0.4 ± 0.4 |
| arachidonic acid | 5 | 19.2 ± 5.7 |
| | 10 | 36.5 ± 9.2 |
| | 20 | 42.7 ± 2.0 |
| oleic acid | 20 | 22.6 ± 2.8 |
| elaidic acid | 20 | 3.7 ± 5.2 |
| linoleic acid | 20 | 27.3 ± 5.2 |
| docosahexaenoic acid | 20 | 34.5 ± 1.8 |

[a] Experimental conditions were as described in Figure 2; $n = 3$.

Other NEFAs have been tested for their capacity to open $K_{ATP}$ channels in insulinoma cells (Table I). Myristic acid ($C_{14}$:0), tetradecadienyl acetate ($C_{14}$:2 *cis*-9,*trans*-11), stearic acid ($C_{18}$:0), *cis*-7,8-epoxy-2-methyloctadecane ($C_{18}$:0) (not shown), and elaidic acid ($C_{18}$:1 *trans*-9) (Table I) have no effect on $K_{ATP}$ channel activity. Conversely (Table I), oleic acid ($C_{18}$:1 *cis*-9), linoleic acid ($C_{18}$:2, *cis*-9,12), and docosa-

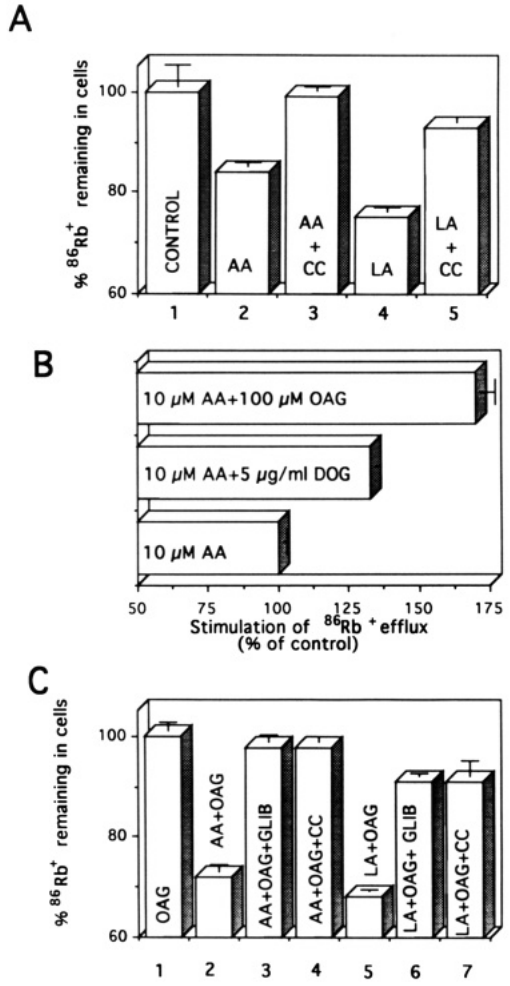


FIGURE 4: Modulation by calphostin C, DOG, and OAG of $^{86}Rb^+$ efflux in HIT $\beta$-cells stimulated by arachidonic and linoleic acids. (A) Calphostin C (CC) inhibits $^{86}Rb^+$ efflux stimulated by arachidonic acid (AA) and linoleic acid (LA). Experimental conditions as described in Figure 2. Column 1, untreated control; column 2, 10 $\mu$M AA; column 3, 10 $\mu$M AA and 1 $\mu$M calphostin C; column 4, 20 $\mu$M linoleic acid; column 5, 20 $\mu$M linoleic acid and 1 $\mu$M calphostin C; $n = 3$. (B) OAG and DOG activate $^{86}Rb^+$ efflux stimulated by arachidonic acid. Efflux caused by 10 $\mu$M arachidonic acid with respect to untreated sample was taken as 100%. Experimental conditions were as described in Figure 2, $n = 3$. (C) Efflux of $^{86}Rb^+$ induced by arachidonic or linoleic acid and OAG is inhibited by calphostin C and glibenclamide (GLIB). Experimental conditions were as described in Figure 2. Column 1, control (in the presence of 100 $\mu$M OAG); column 2, 10 $\mu$M arachidonic acid and 100 $\mu$M OAG; column 3, conditions as for column 2 plus 1 $\mu$M glibenclamide; column 4, conditions as for column 2 plus 1 $\mu$M calphostin C; column 5, 20 $\mu$M linoleic acid plus 100 $\mu$M OAG; column 6, conditions as for column 5 plus 1 $\mu$M glibenclamide; column 7, conditions as for column 5 plus 1 $\mu$M calphostin C; $n = 3$.

hexaenoic acid ($C_{22}$:6 *cis*-4,7,10,13,16,19) were capable of producing $K_{ATP}$ channel opening with different efficacies at an external concentration of 20 $\mu$M. As expected, glibenclamide (100 nM) inhibited the opening effects of these NEFAs (not shown). The activating effects of NEFAs were found to be insensitive to pertussis or cholera toxins, excluding the involvement of G proteins, sensitive to these toxins, which are known to be involved in somatostatin or galanin activation of $K_{ATP}$ channels in insulinoma cells (De Weille et al., 1989; Dunne et al., 1989).

AA and other unsaturated fatty acids are known to be potential activators of subtypes of protein kinase C. Therefore, it was important to see whether the activating effects of NEFAs are suppressed by inhibitors of protein kinase C. Figure 4A shows that $^{86}Rb^+$ efflux induced by activation of $K_{ATP}$ channels by AA or oleic acid is inhibited by calphostin C (1 $\mu$M), a potent and fairly specific inhibitor of kinase C (Kobayashi et al., 1989).

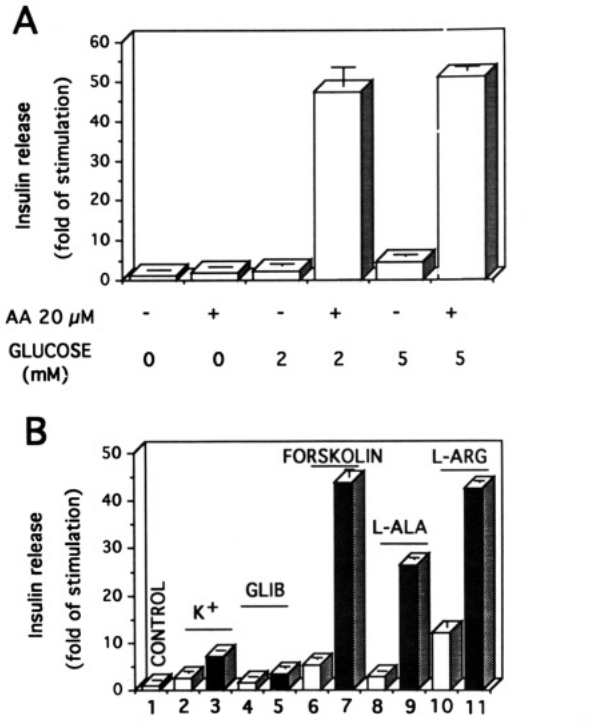


FIGURE 5: Influence of arachidonic acid on insulin release from HIT $\beta$-cells. (A) AA stimulates insulin release induced by glucose. Data are expressed as folds of stimulation of basal insulin release without glucose and AA. The amount of insulin released under these conditions varied from 0.1 to 0.5 ng/mL, $n = 3$. (B) Stimulation by arachidonic acid of insulin release caused by different insulinotropic substances. Data are expressed as folds of stimulation of insulin release in the presence of 2.8 mM glucose (column 1). The amount of insulin release varied from 0.6 to 1.6 ng/mL. Column 2, 15 mM KCl ($K^+$); column 3, 15 mM KCl and 20 $\mu$M arachidonic acid; column 4, 1 $\mu$M glibenclamide; column 5, 1 $\mu$M glibenclamide and 20 $\mu$M arachidonic acid; column 6, 10 $\mu$M foskolin; column 7, 10 $\mu$M forskolin and 20 $\mu$M arachidonic acid; column 8, 10 mM L-alanine (L-ALA); column 9, 10 mM L-alanine and 20 $\mu$M arachidonic acid; column 10, 10 mM L-arginine (L-ARG); column 11, 10 mM L-arginine and 20 $\mu$M arachidonic acid; $n = 3$.

It was also observed that diacylglycerol derivatives DOG and OAG increase $^{86}Rb^+$ efflux induced by arachidonic acid (10 $\mu$M) (Figure 4B). The total $^{86}Rb^+$ efflux induced by AA and diacylglycerols was inhibited both by the $K_{ATP}$ channel blocker glibenclamide (1 $\mu$M) and by the kinase C inhibitor calphostin C (Figure 4C).

Since AA activates $K_{ATP}$ channels and since $K_{ATP}$ channels are involved in the control of insulin secretion (Dunne & Petersen, 1991), it was important to measure the effects of AA on insulin secretion from insulinoma cells. Figure 5 shows that AA (20 $\mu$M) amplified insulin release both in the absence (1.9-fold stimulation over basal release) and in the presence of 2 or 5 mM glucose. Insulin release induced by different secretatogues such as glibenclamide (1 $\mu$M), forskolin (10 $\mu$M), L-alanine (10 mM), or L-arginine (10 mM) or by 15 mM KCl was also amplified by AA (20 $\mu$M). ETYA also produced stimulation of insulin release (not shown).

## Discussion

Fatty acids act as messenger molecules to regulate the function of a number of ionic channels [for a review see Ordway et al. (1991)]. Regulation of ionic channels might involve the fatty acid itself or metabolites of AA from lipoxygenase or cyclooxygenase pathways (Belardetti et al., 1987; Piomelli et al., 1987; Kim & Clapham, 1989; Kim et al., 1989;

Kurachi et al., 1989; Ordway et al., 1989, 1991).

Both $^{86}Rb^+$ efflux experiments and electrophysiological results have shown that AA activates $K_{ATP}$ channels in insulinoma cells. The activatory effect is suppressed by antidiabetic sulfonylureas such as glibenclamide, which are known to be specific blockers of $K_{ATP}$ channels in insulinoma cells (Schmid-Antomarchi et al., 1987) as well as in other cell types (Amoroso et al., 1990). Activation is due to AA itself and not to its metabolites since it is not affected by indomethacin or nordihydroguaiaretic acid which are inhibitors of the cyclooxygenase and lipooxygenase pathways, respectively. Moreover, the nonmetabolized analogue of AA, ETYA, is as potent an activator of the $K_{ATP}$ channel as AA.

Other NEFAs such as oleic, linoleic, and docosahexaenoic acids are able to activate sulfonylurea-sensitive $K_{ATP}$ channels. However, other NEFAs such as myristic, stearic, and elaidic acids are unable to activate $K_{ATP}$ channels under the same conditions.

Activation of $K_{ATP}$ channels by NEFAs is indirect since patch-clamp experiments using excised patches, on which $K_{ATP}$ channel activity can be easily studied, have excluded a direct effect of NEFAs (Figure 3).

The first possibility for this indirect activation of $K_{ATP}$ channels by NEFAs is an uncoupling of mitochondrial respiration which would produce an ATP depletion and, of course, the subsequent opening of $K_{ATP}$ channels. Such a mechanism, which would imply an activation of $K_{ATP}$ channels by saturated NEFAs, which are efficient uncouplers of oxidative phosphorylation (Borst et al., 1962), seems unlikely, since saturated NEFAs do no activate $K_{ATP}$ channels.

The second and most likely mechanism of regulation of $K_{ATP}$ channel activity by NEFAs involves protein kinase C. It is now well-known that unsaturated NEFAs activate protein kinase C in a $Ca^{2+}$- and phospholipid-independent fashion (Naor et al., 1988; Shearman et al., 1991). Moreover, it has been recently observed that there is a synergistic action between free fatty acids and diacylglycerol for the activation of subtypes of protein kinase C (Shinomura et al., 1991). $\alpha$, $\beta$, and $\gamma$ isoforms of protein kinase C are all activated by diacylglycerol and arachidonic, oleic, linoleic, linolic, or docosahexaenoic acid, whereas saturated fatty acids such as palmitic or stearic acid are inactive under the same conditions. This work shows that fatty acids which produce a synergistic activation of protein kinase C with diacylglycerol also activate $K_{ATP}$ channels. Conversely, fatty acids which do not activate protein kinase C do not activate $K_{ATP}$ channels. Moreover, a synergistic effect of diacyglycerols and NEFAs such as AA has been observed for the activation of $K_{ATP}$ channels. Finally, the $K_{ATP}$ channel activatory effects of NEFAs or of NEFAs and diacylglycerol are eliminated by calphostin C, a good inhibitor of protein kinase C (Kobayashi et al., 1989). For all these reasons, NEFA activation of $K_{ATP}$ channels through a pathway involving subtypes of protein kinase C seems to be the most logical explanation. It would be consistent with previous observations showing that phorbol esters, which are well-known activators of protein kinase C, also activate $K_{ATP}$ channels in insulinoma cells (De Weille et al., 1989; Ribalet et al., 1988) [although another report (Wollheim et al., 1988) indicates that phorbol esters can inhibit $K_{ATP}$ channels under different conditions].

Inhibition of $K_{ATP}$ channel activity by high glucose perfusion or by antidiabetic sulfonylureas is known to produce insulin secretion (Dunne & Petersen, 1991), whereas activation of $K_{ATP}$ channels by somatostatin (De Weille et al., 1989), galanin (De Weille et al., 1988; Dunne et al., 1989), or $K^+$ channel activators such as diazoxide (Quast & Cook, 1989; Hamilton & Weston, 1989) is known to decrease insulin secretion. Therefore, it would be expected that activation of $K_{ATP}$ channels by NEFAs would lead to a decreased insulin secretion. Conversely, AA tends to increase insulin secretion from pancreatic islets [this work and Wolf et al. (1991)]. Clearly, then, NEFAs (like phorbol esters (Sharp et al., 1989; Yada et al., 1989) produce insulin release by a mechanism which is independent of $K_{ATP}$ channel activation.

Since AA levels are known to increase very markedly during brain ischemia (Yoshida et al., 1983), it will be important to know whether AA and other NEFAs activate $K_{ATP}$ channels in the brain and particularly in hippocampus (Mourre et al., 1990a,b; Miller, 1990; Politi & Rogawski, 1991), a brain area susceptible to ischemia (Siesjö, 1988). Such a $K_{ATP}$ channel activation could be associated with the massive $K^+$ release from the ischemic brain (Hansen, 1985; Schaeffer & Lazdunski, 1991).

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**Registry No.** ATP, 56-65-5; $K^+$, 7440-09-7; DOG, 2442-61-7; OAG, 84746-00-9; AA, 506-32-1; ETYA, 52642-57-6; oleic acid, 112-80-1; linoleic acid, 60-33-3; docosahexaenoic acid, 6217-54-5; glibenclamide, 10238-21-8; tolbutamide, 64-77-7; insulin, 9004-10-8; protein kinase, 9026-43-1; glisoxepide, 25046-79-1.

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# Interaction of Protein Kinase C with Phosphatidylserine. 1. Cooperativity in Lipid Binding†

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ABSTRACT: The basis for the apparent cooperativity in the activation of protein kinase C by phosphatidylserine has been addressed using proteolytic sensitivity, resonance energy transfer, and enzymatic activity. We show that binding of protein kinase C to detergent–lipid mixed micelles and model membranes is cooperatively regulated by phosphatidylserine. The sigmoidal dependence on phosphatidylserine for binding is indistinguishable from that observed for the activation of the kinase by this lipid [Newton & Koshland (1989) *J. Biol. Chem. 264*, 14909–14915]. Thus, protein kinase C activity is linearly related to the amount of phosphatidylserine bound. Furthermore, under conditions where protein kinase C is bound to micelles at all lipid concentrations, activation of the enzyme continues to display a sigmoidal dependence on the phosphatidylserine content of the micelle. This indicates that the apparent cooperativity in binding does not arise because protein kinase C senses a higher concentration of phosphatidylserine once recruited to the micelle. Our results reveal that the affinity of protein kinase C for phosphatidylserine increases as more of this lipid binds, supporting the hypothesis that a domain of phosphatidylserine is cooperatively sequestered around the enzyme.

The $Ca^{2+}$/lipid-dependent protein kinase C transduces signals that promote phospholipid hydrolysis (Nishizuka, 1986, 1988; Bell & Burns, 1991). Binding of a wide variety of signals to cell surface receptors results in phospholipase C-catalyzed hydrolysis of phosphatidylinositol bisphosphate, producing the water-soluble head group inositol trisphosphate and the lipid backbone diacylglycerol. Both molecules are critical second messengers in the protein kinase C signaling pathway. Inositol trisphosphate mobilizes intracellular $Ca^{2+}$, thus causing protein kinase C, which is present in the cytosol under resting conditions, to bind to the plasma membrane. Diacylglycerol activates the enzyme to phosphorylate substrates as well as to autophosphorylate.

The "translocation" of protein kinase C from the cytosol to the plasma membrane is well documented for many stimuli and cell types (Kraft et al., 1982; Farrar & Anderson, 1985; TerBush & Holz, 1986). Insight into the mechanism of the kinase–membrane interaction has been obtained by studies with model membranes. Bazzi and Nelsestuen have shown

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