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MOLECULAR PHARMACOLOGY, 42:489–498

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Inhibition of Voltage-Gated Ca^{2+} Channels and Insulin Secretion in HIT Cells by the Ca^{2+}/Calmodulin-Dependent Protein Kinase II Inhibitor KN-62: Comparison with Antagonists of Calmodulin and L-Type Ca^{2+} Channels

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Received April 15, 1992; Accepted June 17, 1992

SUMMARY

To probe for the involvement of Ca^{2+}/calmodulin-dependent protein kinase II in the regulation of insulin secretion, the effects of a specific inhibitor of this enzyme, KN-62, on secretagogue-stimulated insulin secretion, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) rise, membrane depolarization, and nutrient metabolism were examined in HIT-T15 cells. KN-62 dose-dependently inhibited insulin secretion induced by a nutrient mixture (10 mM glucose, 5 mM leucine, and 5 mM glutamine) alone or combined with either the Ca^{2+}-mobilizing receptor agonist bombesin or the cAMP-raising agent forskolin in intact cells. KN-62 did not affect Ca^{2+}- or GTP analogue-induced insulin secretion from permeabilized cells, indicating an action at a step before exocytosis. The stimulating effects of nutrients on insulin secretion, $[Ca^{2+}]_i$, and membrane depolarization were potentiated by bombesin. Similarly, bombesin promoted a larger depolarization and $[Ca^{2+}]_i$ rise in the presence of nutrients. This was associated with enhanced Ca^{2+} mobilization and the appearance of sustained $[Ca^{2+}]_i$ elevation. The bombesin-induced membrane depolarization, like the nutrient effect, was inhibited by diazoxide, suggesting that this is due to closure of ATP-sensitive K^+ channels. Bombesin elicited Ca^{2+} influx by both membrane potential-sensitive and -insensitive conductance pathways. KN-62 did not affect Ca^{2+} mobilization and only partially reduced Ca^{2+} entry during the sustained $[Ca^{2+}]_i$ rise in bombesin-stimulated cells. When added before or during the stimulation, KN-62 dose-dependently inhibited nutrient- and KCl-stimulated $[Ca^{2+}]_i$ elevation and Mn^{2+} influx (reflecting Ca^{2+} entry). The calmodulin antagonist CGS 9343B and the L-type Ca^{2+} channel blocker SR-7037 mimicked the inhibitory effect of KN-62 on stimulated insulin secretion and $[Ca^{2+}]_i$ elevation. Membrane depolarization and nutrient metabolism (reduction of a tetrazolium derivative), however, were not altered by KN-62 treatment, indicating that the early coupling events from nutrient metabolism to closure of ATP-sensitive K^+ channels remain operative. These results suggest that KN-62 and the calmodulin antagonist CGS 9343B inhibit Ca^{2+} influx by means of direct interaction with L-type Ca^{2+} channels, which, in turn, causes inhibition of stimulated insulin secretion. Thus, it appears that Ca^{2+}/calmodulin-dependent protein kinase II is not involved in the regulation of insulin secretion.

Insulin secretion from pancreatic β-cells is regulated by the interplay of nutrients, hormones, and neurotransmitters (1, 2). Nutrients such as glucose enter the cells, and their metabolism generates coupling factors, including ATP and NAD(P)H (1–3). Closure of ATP-sensitive K^+ channels promotes membrane depolarization, in turn leading to the opening of voltage-gated Ca^{2+} channels and an increase in $[Ca^{2+}]_i$ (4). Ca^{2+} plays an essential role in the regulation of insulin secretion, because nutrient-induced secretion depends on the presence of extracellular Ca^{2+} and Ca^{2+} itself can trigger insulin release from permeabilized cells (1, 5). However, the mechanism underlying the Ca^{2+} action is still unclear.

CaM is an important mediator of many Ca^{2+}-directed functions (6). Several CaM kinases have been identified; these include CaM kinases I, II, and III, myosin light chain kinase, phosphorylase kinase, and Ins(1,4,5)P_3 3-kinase (7, 8). CaM kinase II is a multifunctional enzyme that consists of α (50-kDa) and β (60-kDa) subunits in different ratios, depending on the developmental stage and the tissue (7). CaM kinase II is

This work was supported by Grant 32.25665.88 (to C.B.W.) from the Swiss National Science Foundation and in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (to H.H.). G.L. is on leave from the Department of Physiology, Jiangxi Medical College, P. R. China.

ABBREVIATIONS: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; BBS, bombesin; bisoxonol, bis(1,3-diethyl thiobarbiturate)trimethineoxonol; CaM, calmodulin; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N, N, N′, N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRBH, Krebs-Ringer bicarbonate-HEPES buffer; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

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most abundant in brain and has a molecular mass of 300–700 kDa (7). Each of the α and β subunits has associating, catalytic, and regulatory domains. The regulatory domain contains the CaM-binding fragment, autoinhibitory region, and autophosphorylation sites (7). When $[Ca^{2+}]_i$ is increased, Ca^{2+}/CaM binds to CaM kinase II, which relieves the autoinhibitory influence and activates the enzyme. Subsequently, autophosphorylation renders the enzyme Ca^{2+} independent (7, 9). The activity may, therefore, be protracted even after transient increases in $[Ca^{2+}]_i$. It is of interest that injection of CaM kinase II into squid giant synapse stimulates neurotransmitter release (10).

The involvement of CaM in insulin secretion has been inferred from studies with CaM antagonists. Glucose-stimulated insulin secretion is inhibited by such antagonists (11–15), which, however, also affect the Ca^{2+} handling of insulin-secreting cells (12–14, 16). The low specificity of these agents and their interference with many CaM-mediated functions prevent precise interpretation of the experimental results. Immunofluorescence staining of rat islets with an antibody against CaM kinase II revealed the presence of the enzyme in the endocrine cells, with the strongest immunoreactivity being observed in the non-β cell region (17). However, the role of CaM kinase II in insulin secretion has not yet been investigated. Recently, a specific CaM kinase II inhibitor, KN-62, has been synthesized (18). It has high selectivity for CaM kinase II, relative to other protein kinases such as kinase A, kinase C, and myosin light chain kinase (9). Unlike CaM antagonists such as trifluoperazine, W7, and CGS 9343B, which bind to CaM, this compound interacts directly with the regulatory domain of CaM kinase II and prevents the autophosphorylation of the enzyme, both *in vitro* and *in vivo* (9).

In the present study, we used KN-62 to probe for the involvement of CaM kinase II in the stimulation of insulin secretion by nutrients and its potentiation by the Ca^{2+}-mobilizing agent BBS and the cAMP-raising agent forskolin. We found that KN-62 inhibited insulin secretion in intact but not in permeabilized HIT cells. The inhibitory effect of KN-62 on insulin secretion was correlated with its inhibition of voltage-gated Ca^{2+} channels, probably of the L-type. The compound did not affect Ca^{2+} mobilization, mitochondrial metabolism, and membrane depolarization. The synergistic effect of nutrients and BBS on membrane depolarization, $[Ca^{2+}]_i$ rise, and insulin secretion was also examined.

Materials and Methods

Cell culture. The insulin-secreting cell line HIT-T15 was originally provided by Dr. A. E. Boyd III (Baylor College of Medicine, Houston, TX). The cells (passages 70–79) were cultured in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. For static incubation experiments, cells were seeded 2 days before experiments, in 96-well microtiter plates (Falcon), at a density of 6–8 $\times$ 10^4 cells/well. For experiments for measurement of $[Ca^{2+}]_i$ and membrane potential, cultured cells were detached by trypsinization and transferred into a spinner flask containing RPMI 1640 medium supplemented with 25 mM HEPES and 1% newborn calf serum, for 3 hr at 37°, as described previously (19).

Insulin secretion from intact cells. For static secretion experiments, cells in microtiter plates were washed twice with modified glucose-free KRBH containing (in mM) 136 NaCl, 4.8 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5 $NaHCO_3$, and 25 HEPES, pH 7.4, supplemented with 0.1% bovine serum albumin. Thereafter, the cells were preincubated for 30 min in the same glucose-free KRBH with 0–1 μM KN-62. The medium was then changed to one containing the various stimuli, and cells were incubated for another 30 min in the continuous presence of 0–1 μM KN-62, at 37°. The supernatants were removed, centrifuged at 4°, and kept at −20° until insulin assay. Attached cells were extracted with acid-ethanol for insulin content determination (20). Insulin was measured by radioimmunoassay, using rat insulin as standard (21).

Insulin secretion from permeabilized cells. For streptolysin-O permeabilization (22), cells seeded in microtiter plates were washed and preincubated for 30 min in glucose-free KRBH, with 0–1 μM KN-62. After two washes with Ca^{2+}-free KRBH and one wash with potassium glutamate buffer (23) at room temperature, cells were rendered permeable by exposure for 5 min at 37° to potassium glutamate buffer containing 1.5 unit/ml streptolysin-O. The permeabilizing solution was then removed. Buffer containing 10.2 mM EGTA and different concentrations of Ca^{2+} and other test agents was then added. Cells were incubated for another 5 min. In some experiments, cells were stimulated by Ca^{2+} during the 5-min permeabilization period. The supernatants were removed and cells were extracted as described above, for measurement of insulin secretion and content, respectively.

For electrical permeabilization, cells maintained in spinner culture were washed and preincubated for 30 min in glucose-free KRBH, with or without 1 μM KN-62. The cells were then washed with Ca^{2+}-free KRBH and exposed to high voltage discharge (30 pulses, 3 kV/cm, 30 μsec) in mannitol buffer, as described previously (23). After centrifugation, cells were preincubated for 15 min at 4° in potassium glutamate buffer containing test agents, to allow equilibration between the intra- and extracellular compartments. The tubes were transferred to 37° and incubated for 5 min. After the incubation was stopped on ice, the tubes were centrifuged. The supernatants were removed and pellets were extracted for determination of insulin secretion and content, respectively. Insulin was measured by radioimmunoassay (21).

Cell permeability was examined by the trypan blue exclusion test. With both methods described above, >95% of cells were permeabilized.

Measurement of $[Ca^{2+}]_i$ and Mn^{2+} influx. Cells were loaded with 1 μM fura-2/acetoxymethyl ester for 30 min at 37°, in RPMI 1640 medium containing 10 mM HEPES and 1% fetal calf serum. Cells were washed with glucose-free KRBH without bovine serum albumin before their transfer into a cuvette (about 1 $\times$ 10^6 cells/ml). Fluorescence was measured in a fluorometer, with excitation and emission wavelengths set at 340 and 505 nm, respectively. Fluorescence of extracellular fura-2 was assessed by addition of 100 μM Mn^{2+} at the beginning and end of each trace, as previously described (19). The signal was computerized and processed mathematically by Excel and corrected for the extracellular fura-2, which increased linearly during the experiment. $[Ca^{2+}]_i$ was calculated using the equation previously described (24).

Ca^{2+} influx was assessed by addition of 200 μM Mn^{2+}, which enters cells via Ca^{2+} channels and quenches intracellular fura-2 fluorescence (19, 25). The changes in the rate of quenching can be taken to indicate changes in the rate of Ca^{2+} influx. The excitation and emission wavelengths were also set at 340 and 505 nm, respectively.

Assessment of membrane potential. After two washes, about 3 $\times$ 10^6 cells were placed in a cuvette containing 2 ml of glucose- and albumin-free KRBH. The fluorescent probe bisoxonol (100 nM) was added. Membrane potential was measured by monitoring the fluorescence, at excitation and emission wavelengths of 540 and 580 nm, respectively (19, 20).

Measurement of cell metabolism by the MTT test. The MTT assay was carried out as described in detail elsewhere for other insulin-secreting cells (26). The formazan crystals formed by reduction of MTT by mitochondrial dehydrogenases were extracted with a solution containing 50% *N,N*-dimethylformamide and 20% sodium dodecyl sulfate.

Statistical analyses. Results are given as mean ± standard error of n experiments and were analyzed by unpaired two-tail Student's t test.

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Materials. The sources of materials used have been indicated elsewhere (19), except the following: forskolin was purchased from Calbiochem (San Diego, CA), BBS from Bachem (Bubendorf, Switzerland), and streptolysin-O from Wellcome Diagnostics (Dartford, UK). CGS 9343B was kindly provided by Dr. A. F. Weitsch, ZYMA SA. (Nyon, Switzerland), and SR-7037 was a generous gift from Dr. E. Niesor, Symphar SA. (Geneva, Switzerland).

Results

Effect of KN-62 on insulin secretion in intact HIT cells. Fig. 1 shows that KN-62 inhibited secretagogue-stimulated insulin secretion in a dose-dependent manner. In control cells, mixed nutrients (10 mM glucose, 5 mM leucine, and 5 mM glutamine) caused a 7.7-fold increase in insulin secretion over 30 min. Inclusion of 0.1 μM BBS and 1 μM forskolin potentiated the mixed nutrient effects 2.5- and 3.5-fold, respectively. When cells were treated with KN-62 during 30-min preincubation and 30-min incubation periods to prevent the CaM kinase II from autophosphorylation, basal insulin secretion was not altered. However, the mixed nutrient-stimulated insulin secretion was inhibited by 24, 68, and 84%, respectively, in 0.1, 0.3, and 1 μM KN-62-treated cells. Under similar conditions, insulin secretion elicited by mixed nutrients plus BBS was reduced by 25, 47, and 87%, whereas that elicited by mixed nutrients plus forskolin was decreased by 24, 50, and 88%, respectively. KN-62 (1 μM) also inhibited high (24 mM) K^+-induced insulin secretion by 89%.

Effect of KN-62 on insulin secretion from permeabilized HIT cells. To examine whether the inhibitory effect of KN-62 on insulin secretion in intact cells is exerted at the final step of exocytosis, insulin secretion from permeabilized cells was measured. Experiments were first performed in streptolysin-O-permeabilized cells. In controls, the basal secretion (at 0.1 μM Ca^{2+}) was 2.8 ± 0.3% of cell content ($n = 5$) over 5 min. GTPγS (100 μM), a poorly hydrolyzable GTP analogue, caused a 4.7-fold increase in insulin secretion (14.3 ± 3.7%, $n = 3$). High Ca^{2+} (10 μM) augmented secretion 14-fold (36.2 ± 8.5%, $n = 5$). When cells were treated with 1 μM KN-62, in glucose-free KRBH, for 30 min and during the 5-min permeabilization and 5-min incubation periods, neither the basal (3.8 ± 1.1%, $n = 5$) nor the stimulated insulin secretion in response to GTPγS (17.2 ± 4.4%, $n = 3$) or high Ca^{2+} (35.3 ± 7.6%, $n = 5$) was significantly different from controls.

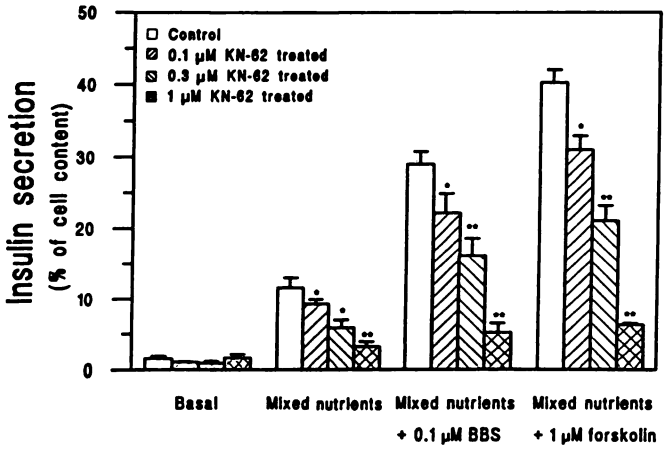

Fig. 1. Effect of KN-62 on secretagogue-stimulated insulin secretion in intact HIT cells. Cells were washed with glucose-free KRBH. After preincubation for 30 min in the same buffer containing 0–1 μM KN-62, at 37°, the medium was changed. The cells were incubated for another 30 min with test agents, in the continuous presence of 0–1 μM KN-62. The supernatants were removed for measurement of insulin secretion, and attached cells were extracted for insulin content determination. Values are mean ± standard error of three to five independent experiments, in sextuplet. Mixed nutrients = 10 mM glucose plus 5 mM leucine plus 5 mM glutamine. *, $p < 0.05$; **, $p < 0.01$, compared with controls.

Because the pores in the plasma membrane produced by streptolysin-O are large (about 20–30 nm) and molecules as large as 480 kDa could leak out, CaM and CaM kinase II may be lost during the 5-min permeabilization and 5-min incubation periods. To study this, two additional series of experiments were performed. In one, GTPγS and high Ca^{2+} were added together with streptolysin-O, and insulin secretion was measured during the 5-min permeabilization period. Under these conditions, the basal secretion was 3.7 ± 0.4% ($n = 6$) and that induced by 2 μM and 10 μM Ca^{2+} was 20.9 ± 2.7% ($n = 6$) and 48.0 ± 4.7% ($n = 6$) of cell content, respectively, in control cells. There was no significant difference in the secretion rates in 1 μM KN-62-treated cells, which were 4.5 ± 0.4% ($n = 6$), 22.5 ± 2.0% ($n = 6$), and 53.4 ± 2.4% ($n = 6$) of cell content, respectively, for basal conditions and after 2 μM and 10 μM Ca^{2+} stimulation.

Another experiment was carried out in electrically permeabilized cells, whose pores are much smaller. Only ions and small molecules (up to 1 kDa) can pass through the holes, whereas proteins are retained in the cells. In this case, basal insulin secretion in control cells was 3.0 ± 0.4% ($n = 4$) of cell content over 5 min. The secretion was increased to 22.4 ± 2.6% ($n = 5$) when cells were stimulated by 10 μM Ca^{2+}. In KN-62 (1 μM)-treated cells, the insulin secretion under basal conditions and initiated by 10 μM Ca^{2+} was 3.4 ± 1.3% ($n = 5$) and 20.4 ± 1.1% ($n = 5$) of cell content, respectively. Thus, KN-62 treatment does not modify the Ca^{2+}-stimulated insulin secretion in streptolysin-O-permeabilized and electrically permeabilized HIT cells, suggesting that CaM kinase II may not be involved in the exocytosis evoked by Ca^{2+} and GTPγS.

Effect of KN-62 on $[Ca^{2+}]_i$ rises. Intracellular free Ca^{2+} is an important regulator of insulin secretion. To assess whether the inhibition of insulin secretion by KN-62 is due to alterations of Ca^{2+} handling, $[Ca^{2+}]_i$ was measured in fura-2-loaded HIT cells.

Pretreatment of cells for 30 min with KN-62, in glucose-free KRBH, inhibited mixed nutrient-induced $[Ca^{2+}]_i$ rises in a dose-dependent manner. In these experiments and in those assessing membrane potential, the final concentrations of added mixed nutrients were 10 mM glucose, 1.5 mM leucine, and 3 mM glutamine, due to the limited solubility of the two amino acids in 100-fold concentrated stock solution. Addition of the mixed nutrients caused a slow $[Ca^{2+}]_i$ increase, with a peak at 2 min (32% above basal) (Fig. 2A; Table 1). Thereafter $[Ca^{2+}]_i$ decreased gradually to near-resting levels. BBS (0.1 μM) added after the mixed nutrients evoked a biphasic rise in $[Ca^{2+}]_i$. The peak $[Ca^{2+}]_i$ was reached within 10 sec and represented a 2.4-fold increase above basal. The sustained $[Ca^{2+}]_i$ increase was 40% above resting levels. Although KN-62 slightly reduced basal $[Ca^{2+}]_i$ levels after 30-min treatment with the compound (see legend to Table 1), it did not alter the resting $[Ca^{2+}]_i$ when added acutely (data not shown). After exposure to 0.1, 0.3, and 1 μM KN-62, the $[Ca^{2+}]_i$ response to mixed nutrients was inhibited by 29, 47, and 94%, respectively (Fig. 2, B–D; Table 1). Under these conditions, the subsequent

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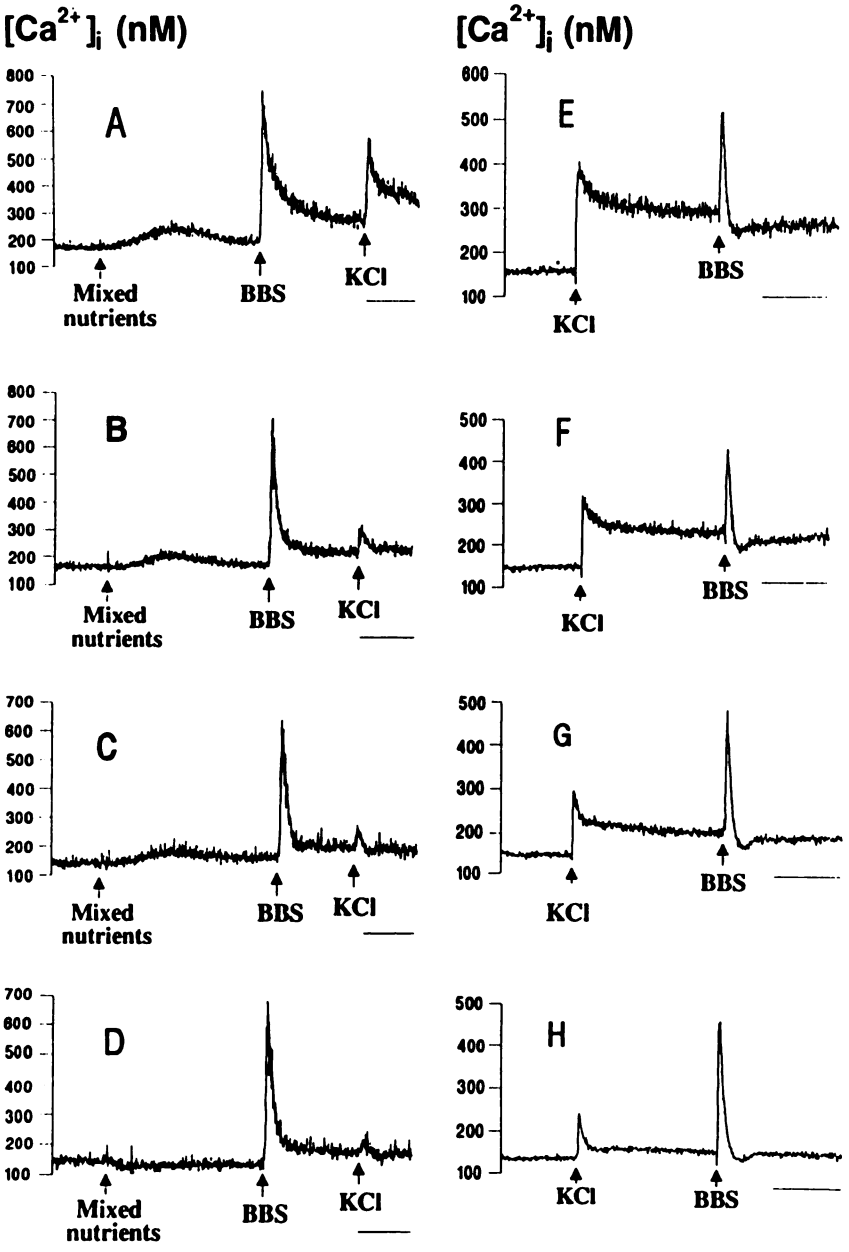

Fig. 2. Effect of KN-62 on $[Ca^{2+}]_i$ rises induced by mixed nutrients, BBS, and KCl in HIT cells. Fura-2-loaded cells were preincubated in glucose-free KRBH containing 0–1 μM KN-62, for 20 min at 20° and 10 min at 37°, before addition of the stimuli. A and E, control cells without pretreatment; B and F, pretreated with 0.1 μM KN-62; C and G, pretreated with 0.3 μM KN-62; D and H, pretreated with 1 μM KN-62. The final concentrations were as follows: mixed nutrients, 10 mM glucose plus 1.5 mM leucine plus 3 mM glutamine; BBS, 0.1 μM; KCl, 24 mM. The *traces* are representative of at least three experiments in each case. *Horizontal bars*, 100 sec.

$[Ca^{2+}]_i$ peak evoked by BBS was not altered, whereas the sustained $[Ca^{2+}]_i$ increase was diminished by 27, 35, and 50%, respectively (Fig. 2, B–D; Table 1).

Similar effects of KN-62 were seen when the cells were depolarized with KCl. Addition of 24 mM KCl caused a 3-fold increase in $[Ca^{2+}]_i$ at peak level (Fig. 2E; Table 1). This $[Ca^{2+}]_i$ peak was inhibited by 39, 62, and 70% after 30-min pretreatment of cells with 0.1, 0.3, and 1 μM KN-62, respectively (Fig. 2, F–H; Table 1). When 0.1 μM BBS was introduced after high KCl, a large $[Ca^{2+}]_i$ transient was elicited, followed by a decrease below the KCl plateau (Fig. 2E). Acute treatment of the cells with 1 μM KN-62 for 3 min exerted similar inhibitory effects on stimulated $[Ca^{2+}]_i$ rises due to mixed nutrients and to KCl (data not shown).

When cells were stimulated by BBS in the absence of mixed nutrients, only a monophasic $[Ca^{2+}]_i$ spike was evoked (Fig. 3A). The peak $[Ca^{2+}]_i$ was 70% of that seen in the presence of nutrients (Table 1). This $[Ca^{2+}]_i$ spike was not affected by KN-62 treatment (Fig. 3B; Table 1). Conversely, BBS potentiated mixed nutrient-induced $[Ca^{2+}]_i$ rises. After 4–5-min stimulation of cells with 0.1 μM BBS, the nutrients increased $[Ca^{2+}]_i$ by 64%, which was significantly higher (2-fold) than in the absence of BBS (Fig. 3A; Table 1). Under these conditions, the mixed nutrient action was inhibited by 34, 69, and 92%, respectively, in 0.1, 0.3, and 1 μM KN-62-treated cells (Table 1; Fig. 3B).

The synergism of mixed nutrients and BBS on $[Ca^{2+}]_i$ was also seen when both stimuli were added simultaneously. In this case a large monophasic $[Ca^{2+}]_i$ rise occurred, reminiscent of the subsequent KCl response (Fig. 3C). However, the peak $[Ca^{2+}]_i$ was like that induced by BBS in the absence of nutrients. In 1 μM KN-62-treated cells, only a $[Ca^{2+}]_i$ transient, like that in Fig. 3B, was evoked by addition of the combination of nutrients and BBS (data not shown).

CGS 9343B, a CaM antagonist, has been reported to inhibit KCl-induced $[Ca^{2+}]_i$ rise in RINm5F cells, another insulin-secreting line (16). We used this agent for a comparison with

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TABLE 1

Effect of KN-62 on $[Ca^{2+}]_i$ rises in HIT cells induced by various stimuli

The basal levels of $[Ca^{2+}]_i$ were (in nM) 157 ± 4 (23), 147 ± 4 (13), 139 ± 2 (12), and 138 ± 4 (19) after 30-min pretreatment of cells in glucose-free KRBH with 0, 0.1, 0.3, and 1 μM KN-62, respectively. $[Ca^{2+}]_i$ was measured as described in Materials and Methods. Values are given as mean ± standard error, and the number of observations is indicated in parentheses.

Condition	Peak $[Ca^{2+}]_i$			
	0[a]	0.1	0.3	1.0
	% of basal			
KCl, 24 mM	303 ± 31 (4)	223 ± 9 (3)	177 ± 14[b] (3)	160 ± 11[b] (4)
Mixed nutrients[c]	132 ± 4 (9)	126 ± 5 (7)	117 ± 3[d] (5)	102 ± 2[b] (10)
Mixed nutrients[e]	164 ± 8 (10)	142 ± 5 (3)	120 ± 2[b] (4)	105 ± 3[b] (5)
BBS, 0.1 μM	269 ± 14 (10)	262 ± 28 (3)	273 ± 23 (4)	297 ± 23 (6)
BBS, 0.1 μM[f]	341 ± 18 (10)	358 ± 18 (8)	361 ± 13 (6)	384 ± 21 (11)
Ionomycin, 1 μM	354 ± 33 (3)			371 ± 18 (3)
Condition	Second phase $[Ca^{2+}]_i$[g]			
	0[a]	0.1	0.3	1.0
	% of basal			
BBS, 0.1 μM	106 ± 2 (10)	106 ± 4 (3)	106 ± 2 (4)	105 ± 2 (6)
BBS, 0.1 μM[f]	140 ± 5 (10)	129 ± 3 (8)	126 ± 2 (6)	121 ± 3[b] (11)

[a] KN-62 treatment (μM).
[b] $p < 0.01$, compared with controls.
[c] Mixed nutrients = 10 mM glucose, 1.5 mM leucine, and 3 mM glutamine.
[d] $p < 0.05$.
[e] Added 4–5 min after 0.1 μM BBS.
[f] Added 4–5 min after mixed nutrients.
[g] Measured 2 min after addition of BBS.

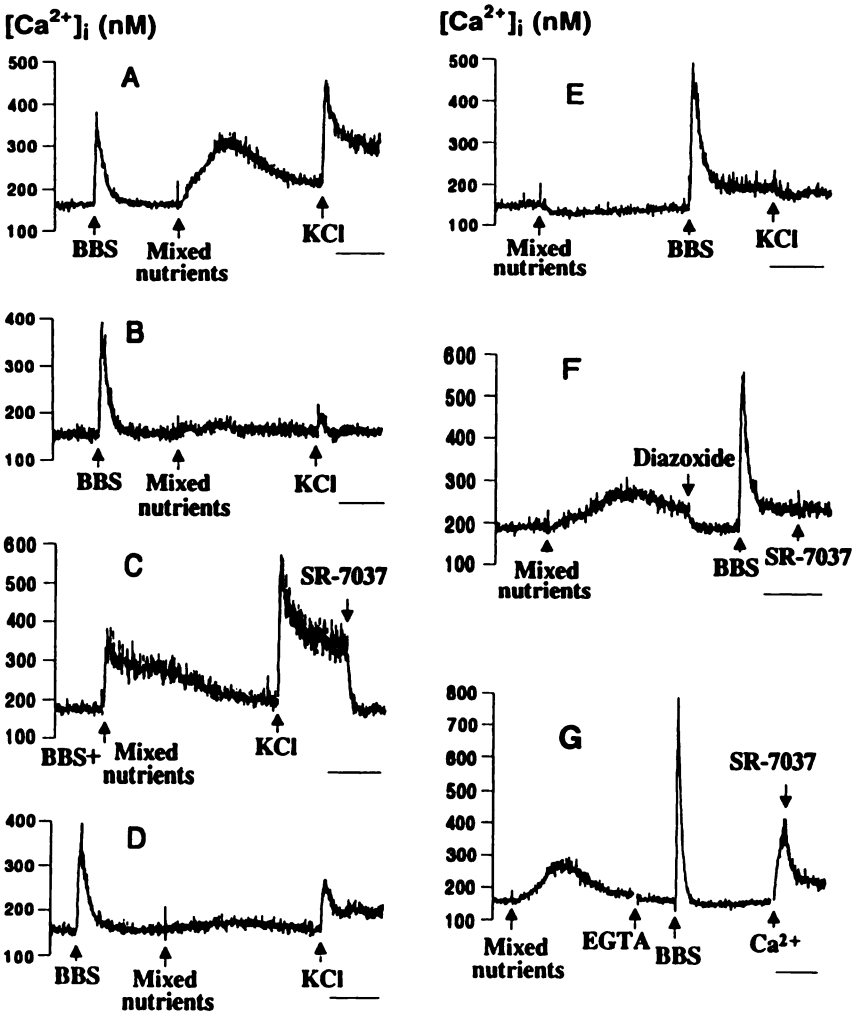

Fig. 3. $[Ca^{2+}]_i$ rises in HIT cells under various conditions. A, C, F, and G, control cells without pretreatment; B, pretreated for 30 min with 1 μM KN-62; D, pretreated for 10 min with 5 μM CGS 9343B; E, pretreated for 10 min with 1 μM SR-7037. The final concentrations were as follows: BBS, 0.1 μM; SR-7037, 1 μM; diazoxide, 200 μM; EGTA, 2 mM; Ca^{2+}, 2 mM; KCl, 24 mM; mixed nutrients, 10 mM glucose plus 1.5 mM leucine plus 3 mM glutamine. The *traces* are representative of at least three experiments in each case. *Horizontal bars*, 100 sec.

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KN-62. Treatment of the cells with 5 μM CGS 9343B for 10 min almost abolished the increase in $[Ca^{2+}]_i$ due to mixed nutrients and inhibited the main part of the $[Ca^{2+}]_i$ rise due to 24 mM KCl (Fig. 3D). However, the $[Ca^{2+}]_i$ response to BBS was not altered. These results suggest that KN-62 and CGS 9343B may act by a similar mechanism, probably by interfering with Ca^{2+} channel function.

The fact that the inhibitory effect of KN-62 on evoked $[Ca^{2+}]_i$ rises is due to blockade of voltage-dependent L-type Ca^{2+} channels is suggested from the results in Fig. 3E. Treatment of the cells for 10 min with 1 μM SR-7037, a potent blocker of L-type Ca^{2+} channels (27), abolished both mixed nutrient and KCl effects on $[Ca^{2+}]_i$. However, as with KN-62, SR-7037 did not alter the first phase and only partially inhibited the second phase of the BBS-induced $[Ca^{2+}]_i$ rise. Similar results were obtained with another L-type Ca^{2+} channel blocker, verapamil, at 20 μM (data not shown). The nature of the remaining, L-channel blocker-insensitive, $[Ca^{2+}]_i$ component was further examined. In one protocol, 200 μM diazoxide, which opens ATP-sensitive K^+ channels (28) and hyperpolarizes cells (see below), was added after mixed nutrients. In this case, diazoxide lowered the nutrient-stimulated $[Ca^{2+}]_i$ rise to basal levels and attenuated the BBS-induced second-phase $[Ca^{2+}]_i$ elevation by about 50% (Fig. 3F). Subsequent addition of SR-7037 did not further decrease $[Ca^{2+}]_i$. In another protocol, the cells were first challenged with mixed nutrients in KRBH containing 1 mM Ca^{2+} (Fig. 3G). EGTA (2 mM) was then added to chelate extracellular Ca^{2+} (<0.1 μM free Ca^{2+}, as measured with a Ca^{2+}-selective electrode). Under these conditions, 0.1 μM BBS elicited only a $[Ca^{2+}]_i$ spike, and no second phase was seen. Restoration of normal extracellular Ca^{2+} by addition of 2 mM Ca^{2+} initiated a $[Ca^{2+}]_i$ rise, which was partially inhibited by 1 μM SR-7037 (Fig. 3G). Even in the presence of EGTA, the BBS-evoked $[Ca^{2+}]_i$ spike was not reduced (385 ± 15% of basal, $n = 4$; see Table 1 for control values). Without nutrient pretreatment, the BBS-elicited $[Ca^{2+}]_i$ transient was smaller and similar to that in the presence of normal extracellular Ca^{2+}, pointing to the potentiating action of nutrients on Ca^{2+} mobilization. These results demonstrate that the initial BBS-induced $[Ca^{2+}]_i$ spike is due to mobilization of Ca^{2+} from intracellular stores, whereas the secondary sustained $[Ca^{2+}]_i$ increase seen in the presence of nutrients is due to Ca^{2+} influx, in part through L-type Ca^{2+} channels and in part through membrane potential-insensitive conductance.

Forskolin (1 μM) caused a transient $[Ca^{2+}]_i$ rise when added after mixed nutrients (Fig. 4A). KN-62 (1 μM) inhibited the forskolin effect (Fig. 4B). The cAMP-raising agent-induced $[Ca^{2+}]_i$ rise could also be blocked by 1 μM SR-7037 (data not shown), implicating L-type Ca^{2+} channels.

Next, we examined whether KN-62 can affect $[Ca^{2+}]_i$ under conditions in which Ca^{2+} channels are already opened. When KN-62 (1 μM) was added to cells that had been depolarized by KCl, there was an immediate decrease in $[Ca^{2+}]_i$, which gradually returned to basal levels. Subsequent addition of SR-7037 (1 μM) did not further lower $[Ca^{2+}]_i$ (Fig. 4C). At 0.1 and 0.3 μM, KN-62 only partially reduced the KCl-evoked $[Ca^{2+}]_i$ rise, and under these conditions SR-7037 caused $[Ca^{2+}]_i$ to return to basal values (data not shown). The time course of the effect of KN-62 on the reduction of $[Ca^{2+}]_i$ differed from that of SR-7037, inasmuch as the latter promoted a more rapid effect (Figs. 4D and 3C).

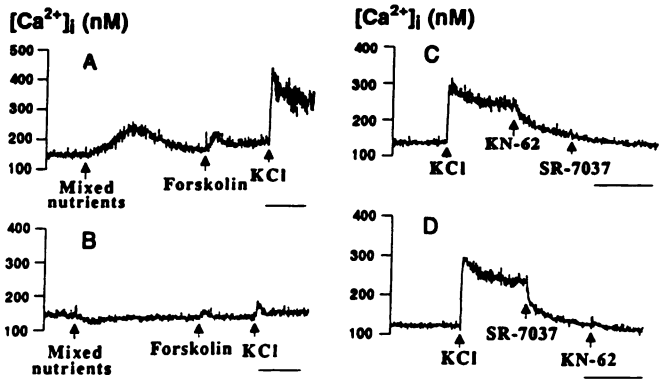

Fig. 4. Effect of KN-62 of forskolin- and KCl-induced $[Ca^{2+}]_i$ rise. A, C, and D, control cells without pretreatment; B, pretreated for 30 min with 1 μM KN-62. The final concentrations were as follows: forskolin, 1 μM; SR-7037, 1 μM; KCl, 24 mM; KN-62, 1 μM; mixed nutrients, 10 mM glucose plus 1.5 mM leucine plus 3 mM glutamine. The *traces* are representative of at least three experiments in each case. *Horizontal bars*, 100 sec.

Finally, the effect of KN-62 on the Ca^{2+} ionophore-elicited $[Ca^{2+}]_i$ rise was examined. Ionomycin (1 μM) caused a sustained $[Ca^{2+}]_i$ increase, with a peak value of 2.5-fold above basal. Pretreatment of cells with 1 μM KN-62 did not alter the ionophore-evoked $[Ca^{2+}]_i$ elevation (Table 1), suggesting that KN-62 selectively affects the voltage-sensitive Ca^{2+} channels.

Effect of KN-62 on Mn^{2+} influx. Ca^{2+} influx was further assessed by using Mn^{2+}, which enters cells through Ca^{2+} channels and quenches intracellular fura-2 fluorescence (19, 25). The changes in the rate of quenching can be taken to indicate the open state of Ca^{2+} channels.

Under resting conditions, there was a slow quenching of fura-2 fluorescence after the addition of 200 μM Mn^{2+}. Depolarization of the cells with 24 mM KCl increased fluorescence transiently (increased $[Ca^{2+}]_i$) and subsequently caused a dramatic increase (7.4-fold) in the rate of quenching (Fig. 5). BBS (0.1 μM) added after KCl elicited a $[Ca^{2+}]_i$ spike. The rate of quenching was then decreased, which fits the previous observation that BBS reduces the KCl-induced $[Ca^{2+}]_i$ rise (see Fig. 2E). At the end of the experiment, permeabilization of the cells with 0.1% Triton X-100 resulted in complete quenching of the fura-2 fluorescence.

Pretreatment of the cells with KN-62 inhibited the KCl-induced Mn^{2+} quenching in a dose-dependent manner (Fig. 5). The increase of quenching rates by KCl was decreased by 27, 55, and 88% in the presence of 0.1, 0.3, and 1 μM KN-62, respectively. As expected, the $[Ca^{2+}]_i$ spikes evoked by subsequent BBS stimulation were affected inversely by KN-62 (Fig. 5). This is because less intracellular fura-2 was quenched by Mn^{2+} when a higher dose KN-62 was present. KN-62 also inhibited the mixed nutrient-induced increase of the quenching rate (3-fold). When the cells were pretreated with 0.1, 0.3, and 1 μM KN-62, the rates were reduced by 45, 59, and 85%, respectively. These results indicate that KN-62 inhibits voltage-dependent Ca^{2+} channels, as demonstrated by measurement of both $[Ca^{2+}]_i$ and Ca^{2+} influx (reflected by the Mn^{2+} quenching).

Effect of KN-62 on depolarization induced by nutrients and BBS. To investigate whether KN-62 inhibits Ca^{2+} channel gating by blocking membrane depolarization, membrane potential was monitored with the fluorescent probe bisoxonol. The results were expressed as percentage of the effect

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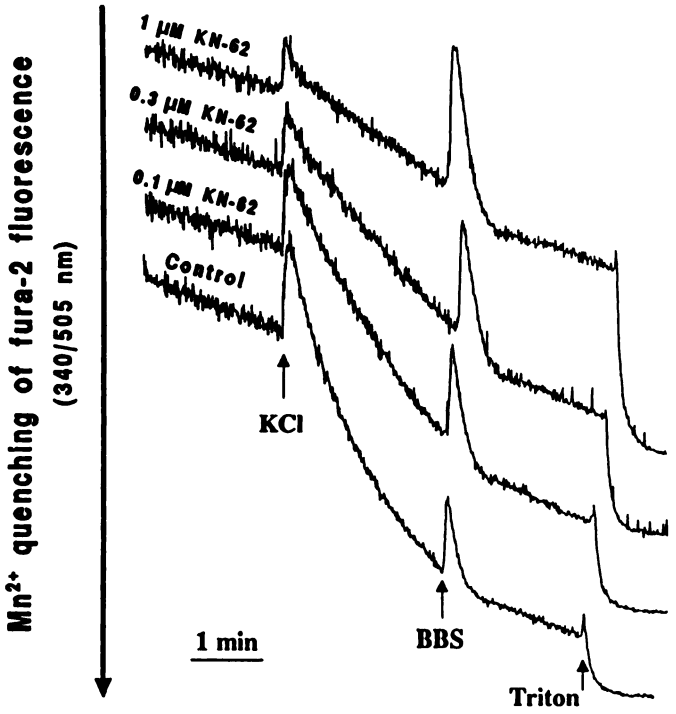

Fig. 5. Effect of KN-62 on Mn^{2+} quenching of intracellular fura-2 fluorescence in HIT cells. Fura-2-loaded cells were preincubated for 20 min at 20° and 10 min at 37° in glucose-free KRBH containing 0–1 μM KN-62. Mn^{2+} (200 μM) was added 2 min before stimulation of the cells with KCl. Excitation and emission wavelengths were set at 340 and 505 nm, respectively. The final concentrations were as follows: BBS, 0.1 μM; KCl, 24 mM; Triton X-100, 0.1%. The traces are representative of at least three experiments in each case.

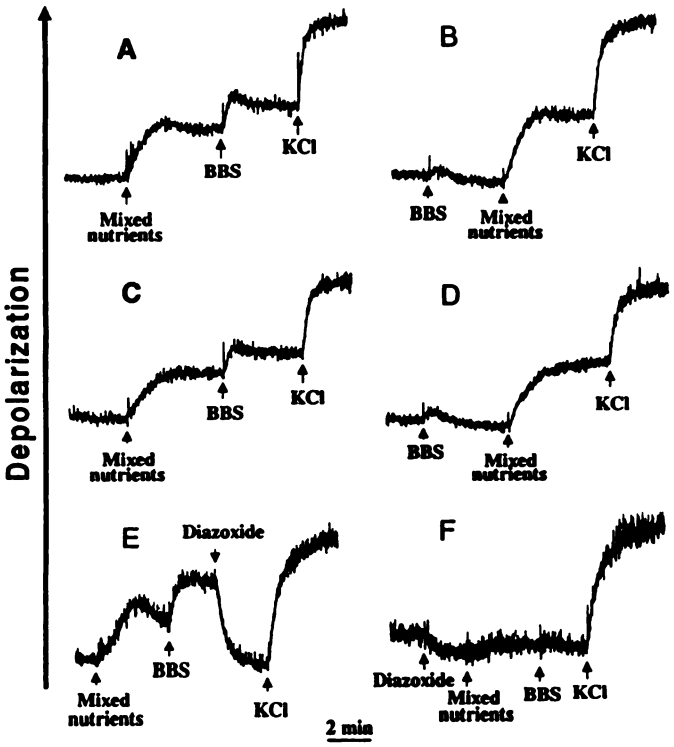

Fig. 6. Effect of KN-62 and diazoxide on membrane depolarization induced by mixed nutrients and BBS in HIT cells. The cells were preincubated for 30 min in glucose-free KRBH, with or without 1 μM KN-62, at 37°. The membrane potential-sensitive fluorescent probe bisoxonol was added 20 min before stimulation of cells, in order to obtain a stable base line. Upward deflections indicate depolarization. A, B, E, and F, control cells without pretreatment; C and D, pretreated with 1 μM KN-62. The final concentrations were as follows: bisoxonol, 100 nM; BBS, 0.1 μM; KCl, 24 mM; diazoxide, 200 μM; mixed nutrients, 10 mM glucose plus 1.5 mM leucine plus 3 mM glutamine. The *traces* are representative of at least three experiments in each case.

of 24 mM KCl added at the end of each trace (relative to the initial fluorescence).

After 30-min preincubation of cells in glucose-free buffer, addition of mixed nutrients (same concentrations as used in the measurement of $[Ca^{2+}]_i$) caused a sustained depolarization, which was further enhanced by 0.1 μM BBS (Fig. 6A). When cells were stimulated by BBS in the absence of nutrients, however, only a marginal depolarization was elicited (Fig. 6B), 6.4 ± 0.8 (n = 4), compared with 51.5 ± 1.3 (n = 6) in the presence of nutrients. Conversely, the depolarization due to mixed nutrients was also potentiated after priming of cells with the Ca^{2+}-mobilizing agent (44.7 ± 2.1, n = 4, and 36.7 ± 2.6, n = 6, in the presence and absence of BBS, respectively; $p < 0.05$) (Fig. 6B). The synergism between nutrients and BBS in membrane depolarization could explain their mutual potentiating action on $[Ca^{2+}]_i$.

Pretreatment of cells with 1 μM KN-62, the dose that markedly inhibited insulin secretion and $[Ca^{2+}]_i$ rise, did not modify the depolarizing action of either nutrients or BBS (Fig. 6, C and D). The values were 6.6 ± 1.5 (n = 3) and 54.0 ± 3.6 (n = 6), respectively, for BBS added before and after nutrients and 35.9 ± 3.6 (n = 6) and 48.2 ± 7.0 (n = 3) for nutrients introduced before and after BBS. These results suggest that KN-62 does not affect the processes by which nutrients and BBS depolarize the cells.

It has been established that glucose depolarizes HIT cells by closing ATP-sensitive K^+ channels (29), whereas the mechanism underlying the effect of BBS is unclear. To investigate this question, we used diazoxide, a known activator of ATP-sensitive K^+ channels (28). When 200 μM diazoxide was added to cells already depolarized by nutrients and BBS, the cells repolarized to prestimulatory levels (Fig. 6E). Likewise, addition of diazoxide before the stimuli caused hyperpolarization and abolished the effect of both nutrients and BBS (Fig. 6F). It thus appears that BBS, like nutrients, depolarizes cells by closure of ATP-sensitive K^+ channels. A similar effect is also seen for vasopressin and ATP in RINm5F cells (30, 31), in which closure of these K^+ channels has been directly demonstrated (31, 32).

Comparison of effects of KN-62, SR-7037, and CGS 9343B on insulin secretion. To investigate whether KN-62 inhibits insulin secretion primarily by blocking voltage-gated Ca^{2+} channels, its effects were compared with those of the L-channel blocker SR-7037 and the CaM antagonist CGS 9343B. As shown in Fig. 7, SR-7037 inhibited mixed nutrient-induced insulin secretion by 90%, compared with 77% inhibition by KN-62 and 39% by CGS 9343B. Insulin secretion due to the combination of mixed nutrients and BBS was also inhibited, in the same order of potency, by the three substances (93%, 85%, and 68%, respectively). When forskolin was used to potentiate mixed nutrient-evoked release, SR-7037 caused 89% inhibition, whereas KN-62 and CGS 9343B reduced secretion by 76% and 49%. Thus, these results suggest that interference with Ca^{2+} influx leads to marked inhibition of insulin secretion and that the three agents under study may all act by this mechanism.

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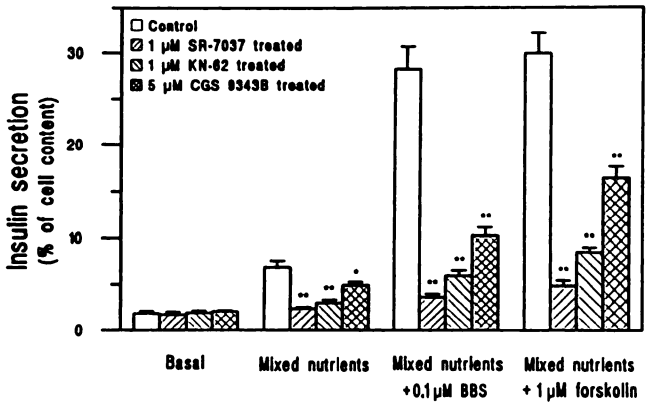

Fig. 7. Comparison of the effects of SR-7037, KN-62, and CGS 9343B on insulin secretion from HIT cells. The cells were washed and preincubated first for 15 min in glucose-free KRBH and then for another 15 min in the same buffer containing 1 μM SR-7037, 1 μM KN-62, or 5 μM CGS 9343B. The medium was changed and the cells were incubated for 30 min with test agents, in the continuous presence of 1 μM SR-7037, 1 μM KN-62, or 5 μM CGS 9343B where appropriate. Mixed nutrients = 10 mM glucose, 5 mM leucine, and 5 mM glutamine. Values are mean ± standard error of two independent experiments, in sextuplet. *, $p < 0.05$; **, $p < 0.001$, compared with controls.

Effect of KN-62 on nutrient metabolism. Nutrient metabolism was monitored by the measurement of formazan generation from MTT, which is catalyzed by mitochondrial enzymes (26). The formazan formation was increased 2.2-fold in response to mixed nutrients (222 ± 13% of basal, $n = 13$). Treatment of cells with 1 μM KN-62 or 10 μM CGS 9343B did not modify the reduction of MTT under either basal or stimulated conditions (data not shown). This suggests that the two compounds do not inhibit insulin secretion by interfering with nutrient metabolism.

Discussion

KN-62 inhibited the stimulated insulin secretion, secretagogue-induced $[Ca^{2+}]_i$ rises, and Mn^{2+} influx with similar concentration dependencies. In all cases, the approximate half-maximal effect was seen at 0.3 μM, which is similar to the IC_{50} for KN-62 inhibition of CaM kinase II in brain homogenates (9). The inhibition of insulin secretion is not due to direct interference with the mechanism of exocytosis, because KN-62 did not alter Ca^{2+}- and GTPγS-stimulated secretion from permeabilized cells. Taken together, these observations suggest that KN-62 blocks Ca^{2+} influx across the plasma membrane, rather than abrogating the action of Ca^{2+}. KN-62 did not affect the $[Ca^{2+}]_i$ increase induced by the Ca^{2+} ionophore ionomycin or the Ca^{2+}-mobilizing effect of BBS, indicating its selective effect on voltage-sensitive Ca^{2+} channels in the plasma membrane. Because nutrient-elicited $[Ca^{2+}]_i$ rise is a consequence of membrane depolarization and the gating of voltage-sensitive Ca^{2+} channels, KN-62 could also act by attenuation of the depolarization. This is most unlikely, because KN-62 failed to affect nutrient-evoked increases in bisoxonol fluorescence. Similarly, there was no alteration of nutrient metabolism as assessed by reduction of MTT, a method thought to reflect the activity of succinate dehydrogenase and other mitochondrial dehydrogenases (26).

It thus appears that the blockade of voltage-sensitive Ca^{2+} channels is the primary action of KN-62 in insulin-secreting HIT cells. This idea was supported by the results obtained with the L-channel blocker SR-7037 (27), which were qualitatively very similar to those seen with KN-62, in terms of $[Ca^{2+}]_i$ rise, Mn^{2+} influx, and insulin secretion. Moreover, neither KN-62 nor SR-7037 changed the rapid $[Ca^{2+}]_i$ transient elicited by BBS. This transient is due to Ca^{2+} mobilization from $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores, inasmuch as it was still present when extracellular Ca^{2+} was chelated. BBS has previously been shown to activate phospholipase C in these cells (19, 33). In contrast, the second phase of BBS-induced $[Ca^{2+}]_i$ elevation, which is due to Ca^{2+} influx, was partially inhibited by both KN-62 and SR-7037. The escape from complete inhibition suggests that not only L-channels are activated during this phase.

Forskolin potentiated nutrient-induced insulin secretion to a similar extent as did BBS, although it caused only a small $[Ca^{2+}]_i$ rise. The inhibition of nutrient- plus forskolin-stimulated insulin release by the L-channel blocker SR-7037 indicates that the main action of cAMP is the sensitization of the secretory process to Ca^{2+}, as proposed previously (5, 34). KN-62 does not inhibit cAMP-dependent protein kinase (9). However, it exerted the same inhibitory effect on forskolin-potentiated insulin secretion as did SR-7037, again pointing to a similar mode of action for the two agents.

The question arises whether KN-62 blocks L-type Ca^{2+} channels by inhibition of CaM kinase II. It has indeed been shown that L-channels from skeletal muscle can be phosphorylated by CaM kinase II (35). But, in contrast to phosphorylation by protein kinase C and cAMP-dependent protein kinase, this did not increase channel function (35). Our results in intact cells do not favor the hypothesis that KN-62 blocks Ca^{2+} channels by acting on CaM kinase II. Thus, when $[Ca^{2+}]_i$ was increased to a steady state during K^+ depolarization, KN-62 caused an immediate decrease in $[Ca^{2+}]_i$. Under these conditions, CaM kinase II should be fully activated and, consequently, insensitive to inhibition by KN-62, as has been shown in brain homogenates (9). Recently, KN-62 was reported to inhibit K^+-induced phosphorylation of tyrosine hydroxylase in intact PC12 cells (36). However, this effect could also be due to inhibition of Ca^{2+} entry. In another study, KN-62 was found to block Ca^{2+}-activated Cl^- current stimulated both by receptor agonists and by $Ins(1,4,5)P_3$ (37). Based on these results, it was concluded that CaM kinase II is involved in the Ca^{2+}-induced Cl^- channel activation. An alternative explanation, also applicable to our findings, is that KN-62 could interact directly with both Ca^{2+} and Cl^- channels.

It has been shown previously that CaM antagonists inhibit glucose- or K^+-induced $^{45}Ca^{2+}$ uptake (12–14) and insulin secretion in pancreatic islets (11–15). Such substances, including CGS 9343B, also attenuated the $[Ca^{2+}]_i$ rise evoked by K^+ in insulin-secreting RINm5F cells (16). In the present study, CGS 9343B inhibited the $[Ca^{2+}]_i$ rise due to both K^+ and nutrients, albeit with less potency than KN-62 and SR-7037. Despite this apparent similarity between KN-62 and CGS 9343B, it is highly unlikely that KN-62 acts as a CaM antagonist, because it does not bind to this protein (9). CaM antagonists have been reported to affect Ca^{2+} currents in chick ventricular cells (38), snail neuron (39), and sea urchin sperm (40); this is thought to reflect a direct action of the drugs on the channels. Conversely, it has also been shown that some Ca^{2+} channel blockers bind to CaM (38). These findings and our results suggest that both

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CaM antagonists and KN-62 interfere with the function of voltage-sensitive Ca^{2+} channels in a CaM-independent manner.

In this study, we observed clear potentiation between nutrient stimuli and the Ca^{2+}-mobilizing agonist BBS for membrane depolarization, $[Ca^{2+}]_i$ rise, and insulin secretion. When BBS was present, nutrients evoked a larger $[Ca^{2+}]_i$ elevation, comparable to the larger depolarization under these conditions. Nutrients also potentiated BBS-induced $[Ca^{2+}]_i$ rise. In this case, two effects were observed, a more marked $[Ca^{2+}]_i$ transient (due to increased Ca^{2+} mobilization) and the appearance of sustained $[Ca^{2+}]_i$ elevation (due to Ca^{2+} influx). A similar effect of nutrients on $[Ca^{2+}]_i$ in response to other Ca^{2+}-mobilizing agonists has been reported in HIT (41) and RINm5F cells (42). The mechanism underlying the increased Ca^{2+} mobilization is not clear. There is no report that phospholipase C-activating receptor agonists cause more Ins(1,4,5)P_3 generation in the presence of nutrients. Although glucose can stimulate Ins(1,4,5)P_3 production, most, if not all, of this effect is dependent on the Ca^{2+} influx (43). In our study, nutrients still exerted the same potentiation of BBS-evoked $[Ca^{2+}]_i$ rise when Ca^{2+} influx was blocked by SR-7037 or KN-62, indicating that this effect depends on the nutrient metabolism itself, rather than on other actions. Hellman *et al.* (44) found that Ins(1,4,5)P_3 released more Ca^{2+} from permeabilized islet cells after pretreatment with glucose, possibly due to increased incorporation of Ca^{2+} into the Ins(1,4,5)P_3-sensitive pool. Alternatively, nutrient metabolism might produce factors that enhance the action of Ins(1,4,5)P_3 (45). The BBS-induced second phase of $[Ca^{2+}]_i$ rise is due to Ca^{2+} influx via both membrane potential-sensitive and -insensitive conductance. The former can be explained by the enhanced depolarization, but it is not clear how nutrients facilitate the activation of the latter. This Ca^{2+} conductance pathway could be similar to receptor-mediated Ca^{2+} influx in other cells, the mechanism of which is under debate (46–49).

In conclusion, although KN-62 inhibited insulin secretion in the present study, this cannot be taken as unequivocal evidence for the involvement of CaM kinase II in the triggering of insulin secretion. The main action of this agent appears to be the blockade of voltage-sensitive Ca^{2+} channels. The failure of KN-62 to inhibit Ca^{2+}-stimulated insulin secretion in permeabilized cells argues against a role for CaM kinase II in the control of exocytosis.

Acknowledgments

We are grateful to A.-S. Annen and D. Nappey for their skillful technical assistance. We are also indebted to Dr. M. Prentki for helpful discussions and to J. Gunn for editorial assistance.

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