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Biochimica et Biophysica Acta 1310 (1996) 343–347

BBA Biochimica et Biophysica Acta

KN-62, a calcium/calmodulin-dependent protein kinase II inhibitor, inhibits high potassium-stimulated prolactin secretion and intracellular calcium increases in anterior pituitary cells

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Received 28 June 1995; revised 12 September 1995; accepted 17 October 1995

Abstract

In isolated rat anterior pituitary cells, KN-62 (10 μM), an isoquinoline sulfonamide inhibitor of calcium/calmodulin-dependent protein kinase II, inhibited high KCl(50 mM)-stimulated prolactin secretion almost completely, with an IC_{50} of 95 nM. KN-62 inhibited TRH-induced prolactin secretion less effectively. KN-04, a compound that is over 100-fold less active in inhibiting purified calcium/calmodulin-dependent protein kinase II, also inhibited high KCl-stimulated prolactin secretion with an IC_{50} of 500 nM. KN-62 and KN-04 (10 μM) both inhibited high KCl-stimulated increases in intracellular Ca^{2+} concentrations. We conclude that KN-62 and KN-04 inhibit activation of voltage-dependent calcium channels in anterior pituitary cells either directly or indirectly.

Keywords: Calcium; Calmodulin; Protein kinase; Prolactin; TRH; Calcium channel

KN-62(1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) belongs to the isoquinolone sulfonamide series of protein kinase inhibitors [1,2]. Protein kinase assays using purified kinases showed that KN-62 inhibited the activity of CaM kinase II activity with high potency ($K_i = 0.9$ μM), but had no significant effect on the activity of cyclic AMP-dependent protein kinase, protein kinase C and myosin light chain kinase [2]. CaM kinase II has a wide tissue distribution and may be an important component in many calcium messenger systems [3–5]. As a result, KN-62 has been used in intact cells as a tool to investigate the role of this kinase and has been found to inhibit a whole range of physiological processes, including GABA release into cerebrospinal fluid [6], phosphorylation and activation of tyrosine hydroxylase stimulated by high potassium concentrations (56 mM) in PC12 cells [7], long-term potentiation in the hippocampus [8], cholinergic-stimulated secretion in parietal cells [9], insulin secretion in pancreatic islet β cells, RINm5F cells, and HIT cells [10–12], neurite outgrowth in neuroblastoma cells [13], and the rate of spontaneous beating in cultured fetal mouse cardiac myocytes [14].

We investigated effects of KN-62 on prolactin secretion and increases in $[Ca^{2+}]_i$ stimulated by high potassium concentrations and TRH. We also used KN-04, (N-1(1-1(P-(-isoquinolinsulfonyl)benzyl)-2-(4-phenylpiperazinyl)-ether)-5-isoquinolinsulfonamide), a compound that does not inhibit CaM kinase II activity.

1. Materials and methods

Isolation of rat anterior pituitary cells. Rat anterior pituitary cells were isolated from Sprague-Dawley female retired breeders (8–9 months old) by sequential digestion with trypsin and pancreatin [15].

Prolactin secretion. Isolated cells were cultured in a 1:1 mixture of Dulbecco's modified medium and Ham's F10 medium supplemented with 15% horse serum in a humidi-

Abbreviations: CaM kinase II, calcium/calmodulin-dependent protein kinase II; TRH, thyrotropin-releasing hormone; $[Ca^{2+}]_i$, concentration of intracellular calcium ion.

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SSDI 0167-4889(95)00170-0

fied atmosphere of 95% air and 5% CO_2 for three days. Then cells were washed with Ringer's solution before incubating with Ringer's solution plus KN-62 or KN-04 at the indicated concentrations for 60 min. The Ringer's solution had the following composition in mM: NaCl 120, KCl 4.8, KH_2PO_4 1.2, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgSO_4$ 1.0, glucose 4, HEPES 10 (pH 7.4), and 1 mg/ml bovine serum albumin. The medium was changed to fresh medium containing KN-62 or KN-04 and the stimulus for prolactin release (KCl 50 mM or TRH 0.1 μM); medium and cells were collected after a 10-min incubation. It takes 2 h or more before newly synthesized prolactin is secreted from primary cultures [16], so the effects measured with this assay are primarily on release of previously synthesized hormone. Prolactin in the cells and medium was measured using radioimmunoassay reagents supplied by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development and the US Department of Agriculture. Prolactin secretion was expressed as the percent in the medium of total (medium plus intracellular) in the culture. All data are expressed in means ± S.E.M.; statistical differences were determined by the multiple *t*-test, and $P < 0.05$ taken as significant.

Indo-1 fluorescence measurements of $[Ca^{2+}]_i$. Freshly isolated cells were loaded with indo-1 by incubating with indo-1 AM (10 μM) for 30 min at 37 C [15,17]. Fluorescence was measured in an SLM 4000C spectrofluorometer with excitation at 350 nm and emission detected at 390 nm and 475 nm. Cells were perifused in Ringer's buffer. KCl, 100 mM, was given in pulses both before and after KN-62 or KN-04 treatment. KN-62 or KN-04 was first dissolved in DMSO:ethanol (1:1, v:v) at 1 mM, and then diluted in perifusion buffer to 10 μM. In control experiments, only vehicle (DMSO:EtOH, 0.5% each) was added. In all experiments the ratio of f390nm/f475nm was recorded, and subsequently normalized so that the ratio at time zero was 1.00.

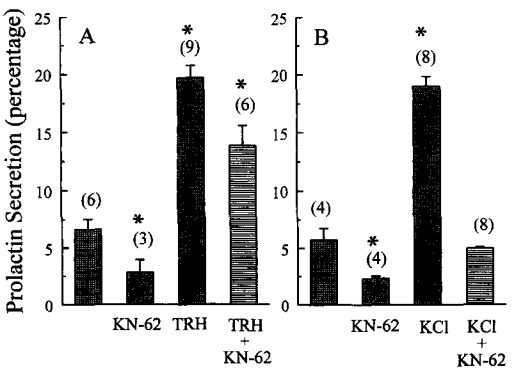

Fig. 1. Effect of KN-62 (10 μM) on prolactin release stimulated by TRH (0.1 μM, Panel A) or KCl (50 mM, Panel B). The data are the mean ± S.E.M. of independent experiments, and the number of experiments is given in brackets. An asterisk above the bar indicates that the value is significantly different than the control ($P < 0.05$).

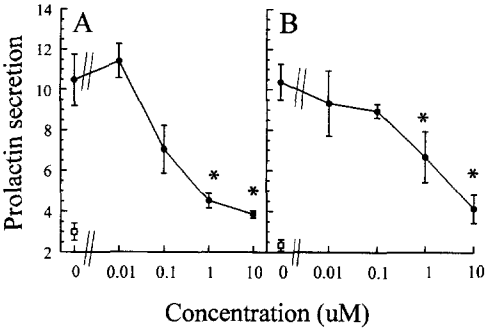

Fig. 2. Effect of KN-62 (Panel A) and KN-04 (Panel B) on prolactin release stimulated by KCl (50 mM). The data are the mean ± S.E.M. of 3 independent experiments. Filled symbols represent results from cells stimulated with KCl, and open symbols, controls with no KCl or KN treatment. An asterisk above the point indicates that the value is significantly different than the value from cells with KCl and no KN-62 or KN-04 ($P < 0.05$).

Materials. Indo-1 AM was purchased from Molecular Probes (Eugene, OR). TRH was from Abbott Laboratories (North Chicago, IL). KN-62 and KN-04 were synthesized as before [2,6].

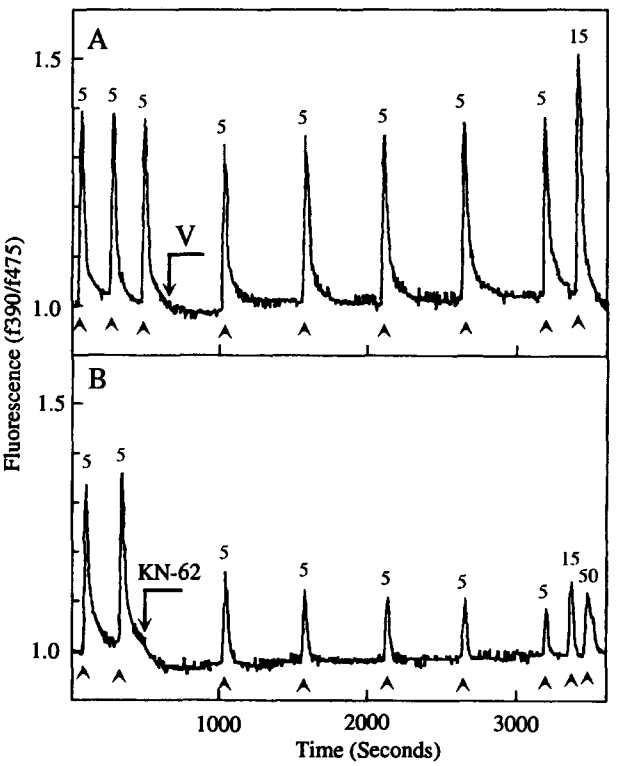

Fig. 3. Effect of KN-62 on KCl-induced increases in $[Ca^{2+}]_i$ in anterior pituitary cells. KCl (100 mM) pulses were given as indicated by the arrowheads under the tracing; the duration in seconds of each pulse is indicated by the numerals above each peak. Vehicle (V) or KN-62 (10 μM) was given as indicated and was present until the end of the experiment. Both trace A and trace B are representative of 3 identical experiments.

2. Results

In primary cultures of anterior pituitary cells, TRH (0.1 μM) stimulated prolactin secretion from a basal level of 6.60 ± 0.93 (percentage of total prolactin in the cultures) to 19.69 ± 1.05 ($P < 0.05$, Fig. 1A). KN-62 (10 μM) alone reduced basal secretion to 2.83 ± 1.10 ($P < 0.05$); TRH-stimulated secretion still occurred in the presence of KN-62, but was reduced to 13.83 ± 1.71 ($P < 0.05$). At least part of the inhibition of TRH-induced secretion may be accounted for by the decrease in basal secretion. High KCl concentrations (50 mM) also stimulated prolactin secretion (from 5.74 ± 1.0 to 19.01 ± 0.84, $P < 0.05$), but this stimulation was completely inhibited by KN-62 (to 4.94 ± 0.12, Fig. 1B).

The effect of KN-62 on KCl-induced prolactin secretion was dose-dependent, almost completely blocking stimulation at 1 μM (Fig. 2A). These concentrations are similar to those that inactivate CaM kinase II in vitro [14]. KN-04, however, which does not inhibit CaM kinase II, does inhibit KCl-induced prolactin secretion (Fig. 2B). KN-04 was less effective than KN-62; the half-maximal inhibitory concentrations were 500 nM and 95 nM, respectively (Fig. 2).

High KCl concentrations depolarize the cells and cause calcium entry through voltage-dependent calcium channels; in anterior pituitary cells, the rise in $[Ca^{2+}]_i$ is completely dependent on extracellular Ca^{2+} [17]. We examined whether KN-62 could affect the increase in $[Ca^{2+}]_i$ in perifused cells. Data in Fig. 3 show that 5-s pulses of KCl (100 mM) could elicit repetitive increases in $[Ca^{2+}]_i$, represented by the fluorescence ratio f390/f475. The vehicle alone did not affect the $[Ca^{2+}]_i$ spikes (Fig. 3A), but KN-62 (10 μM) was inhibitory (Fig. 3B). KN-62 was so effective that a 50-s pulse at the end of the perifusion elicited an increase in the f390/f475 ratio that was only 37% of a 5-s pulse before KN-62 addition (Fig. 3B).

KN-62 (10 μM) could not completely abolish KCl-induced f390/f475 increases, and more extensive recruitment of the voltage-dependent calcium channels was able to overcome the KN-62 inhibition to some extent (Fig. 4). The magnitude of the increase in the f390/f475 ratio by KCl pulses depended on the duration of the pulse. Pulses of 2, 5, and 15 s stimulated increasing spikes of $[Ca^{2+}]_i$.

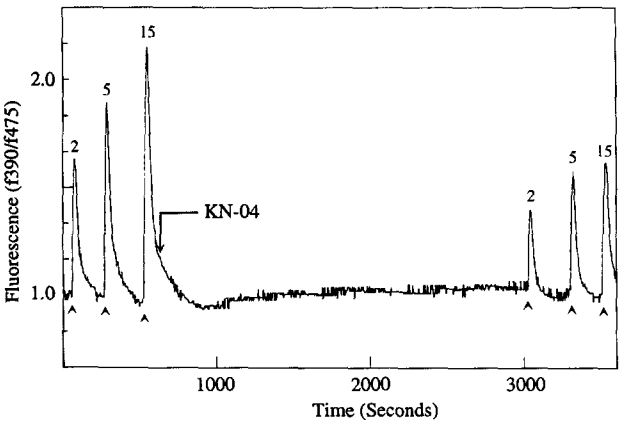

Fig. 5. Effect of KN-04 on KCl-induced increases in $[Ca^{2+}]_i$ in anterior pituitary cells. KCl (100 mM) pulses at 2, 5, and 15 s were given as indicated by the arrowheads. KN-04 (10 μM) was given at the indicated time and was present until the end of the experiment. This trace is representative of three similar experiments.

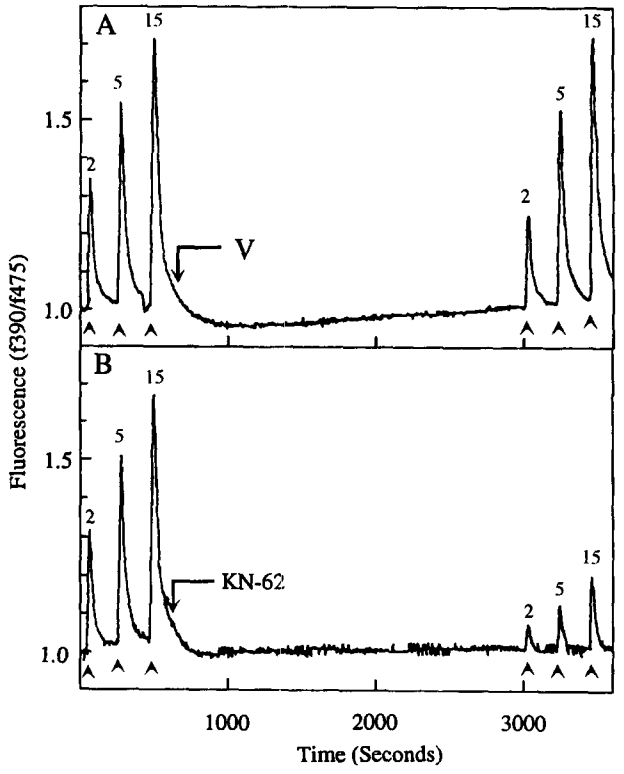

Fig. 4. Effect of KN-62 on KCl-induced increases in $[Ca^{2+}]_i$ in anterior pituitary cells. KCl (100 mM) pulses at 2, 5, and 15 s were given as indicated by the arrowheads. The cells were perifused either with vehicle (V) alone or with KN-62 (10 μM). Trace A is representative of 3 and trace B of 5 identical experiments.

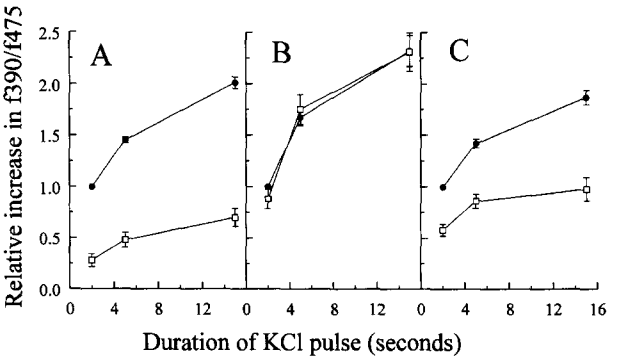

Fig. 6. The effect of KN compounds on KCl-induced $[Ca^{2+}]_i$ increases. Data from the experiments were combined by normalizing the increases in f390/f475 to the first 2-s KCl pulse; the means ± S.E.M. of the increases in the ratio that occurred with subsequent pulses are shown. Closed symbols represent increases with no additions and open symbols represent increases in the presence of KN-62 (Panel A, $n = 5$), vehicle (Panel B, $n = 3$), or KN-04 (Panel C, $n = 3$).

Neither the magnitude nor the duration of the $[Ca^{2+}]_i$ increase was affected by vehicle alone (Fig. 4A). KN-62 markedly diminished KCl-induced $[Ca^{2+}]_i$ increases, but increasing the duration of the pulse did still increase $[Ca^{2+}]_i$ (Fig. 4B).

KN-04 had a similar effect on KCl-induced $[Ca^{2+}]_i$ increases (Fig. 5). KN-04 (10 μM) inhibited $[Ca^{2+}]_i$ spikes of 2, 5, and 15 s pulses. The results of all the experiments with this time course are summarized in Fig. 6. KN-04, like KN-62, inhibited the increase in $[Ca^{2+}]_i$, but was not quite as effective as KN-62.

3. Discussion

In rat pituitary cells, we found that 10 μM KN-62 and KN-04 reduced the increase in $[Ca^{2+}]_i$ caused by activating voltage-dependent Ca^{2+} channels. Increases in $[Ca^{2+}]_i$ trigger prolactin release and KN-62 and KN-04 also reduced prolactin release triggered by activating voltage-dependent Ca^{2+} channels. These compounds did not reduce TRH-induced prolactin release to the same extent, and they had no effect on increases in $[Ca^{2+}]_i$ caused by TRH pulses (data not shown). Although TRH stimulates Ca^{2+} entry [15], it also releases Ca^{2+} from intracellular stores [18], and causes formation of diacylglycerol [19]. Under the conditions of our experiments, these latter two factors may be of primary importance in contributing to prolactin release stimulated by TRH.

One explanation for our data is an action of CaM kinase II on calcium channels. If CaM kinase II regulates voltage-dependent Ca^{2+} channels to increase their activity in certain tissues, then decreasing the activity of CaM kinase II with inhibitors will decrease the ability of the enzyme to activate the channels. The chief argument against this model is that KN-04 has the same effect as KN-62, and KN-04 is not an effective inhibitor of purified CaM kinase II. KN-62 (1 μM) inhibited purified CaM kinase II over 90%, and the K_i was 0.1 μM [14]. KN-04 (10 μM) caused only 20% inhibition and the K_i was 100 μM [14]. We previously demonstrated that lactotrophs have CaM kinase II by immunoblots and immunocytochemistry [20]. It is possible that there is modification of the enzyme in pituitary cells to make it more sensitive to KN-04. We attempted to test this by measuring the effects of KN-04 and KN-62 in homogenates of pituitary cells, using the phosphorylation of a peptide substrate as an assay [21]. We were unable to detect any significant inhibition by either compound under a variety of conditions. Although the pituitary cells may contain forms of CaM kinase II that cannot be inhibited by KN-62, it is also possible that the amount of homogenate we needed to use to detect activity contained enough endogenous calmodulin to competitively inhibit the action of KN-62 [2]. The concentration of KN-62 that we can use is limited by solubility and the effects of DMSO on the homogenate, and therefore these experiments with homogenates are not conclusive. The results in intact cells with KN-04, however, indicate that, if CaM kinase II is involved, the enzyme does not have the same inhibitor specificity in lactotrophs as it does in other tissue.

Use of KN-04 has not been as widespread as KN-62; when it has been used it usually has had no effect [6,10,14,22–24]. There was a small effect on insulin release; 5 μM KN-62 inhibited glucose-stimulated insulin secretion 75%, and 10 μM KN-04 inhibited this stimulation about 25% [10]. The data so far therefore indicate that hormone release from lactotrophs and possibly β-cells of pancreatic islets are sensitive to KN-04, but muscle contraction and neurotransmitter release are not.

The simplest explanation for the decrease in K^+-stimulated prolactin release and $[Ca^{2+}]_i$ caused by KN-62 and KN-04 is that these compounds affect the activity of voltage-dependent Ca^{2+} channels in anterior pituitary cells. KN-62 has a similar effect on insulin release as it does on prolactin release. In HIT insulin-producing cells, KN-62 inhibits K^+-stimulated insulin release and increases in $[Ca^{2+}]_i$ [12]. KN-62 did not affect the increase in $[Ca^{2+}]_i$ caused by bombesin in these cells [12]. The authors suggest that KN-62 directly inhibited L-type Ca^{2+} channels. More recent investigations, however, have indicated KN-62 does not inhibit Ca^{2+} channels in neurons and myocytes [23,25–27]. Therefore, at this time there is no evidence for a direct effect on Ca^{2+} channels.

The results of KN-62 on hormone release in lactotrophs differ from effects on muscle contraction and neurotransmitter release in the inhibition of $[Ca^{2+}]_i$ increases caused by voltage-dependent Ca^{2+} channels. This tissue specificity may be due to differences in Ca^{2+} channels. Pituitary cells have at least two types of calcium channels, L and T channels [28,29]. T channels, activated at low membrane potentials, are not well characterized, but L channels, high voltage-activated channels, are made of oligomeric complexes of four different subunits, α_1, a complex of α_2 and δ, β and γ. There are at least 6 genes coding for α_1 subunits and 4 for β subunits; further diversity based on splice variants and post-translational processing lead to functional differences [30]. Tissue specificity in the channels may explain the effects on $[Ca^{2+}]_i$. Alternatively, the tissue specificity of the KN-62 may be that pituitary cells and possibly β-cells of the pancreas have some process sensitive to KN-62 and KN-04 that indirectly inhibits Ca^{2+} channel activity.

Acknowledgements

This work was supported by NIH Grant HD 11487.

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