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The Ca^{++}/Calmodulin-Dependent Protein Kinase II Inhibitors KN62 and KN93, and Their Inactive Analogues KN04 and KN92, Inhibit Nicotinic Activation of Tyrosine Hydroxylase in Bovine Chromaffin Cells

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The possible role of Ca^{++}/calmodulin-dependent protein kinase II (CAM-K-II) in the nicotinic activation of tyrosine hydroxylase in intact cultured bovine adrenal chromaffin cells has been investigated. Over the concentration range 3–30 μM, KN62, a specific CAM-K-II inhibitor, inhibited basal tyrosine hydroxylase activity and the activity stimulated by nicotine or K^+ depolarisation. KN04, a structural analogue of KN62 which does not inhibit CAM-K-II, produced an identical concentration-dependent inhibition of basal and nicotine-stimulated tyrosine hydroxylase activity. Another CAM-K-II inhibitor, KN93, also inhibited nicotine and K^+ stimulation of tyrosine hydroxylase activity; however, an inactive analogue of KN93, KN92, mimicked these effect. The results suggest that the inhibition of nicotine- and K^+-stimulated tyrosine hydroxylase activity by KN62 and KN93 is not due to their ability to inhibit CAM-K-II.

Tyrosine hydroxylase (TOH; EC1.14.16.2) is the rate-limiting enzyme in the biosynthesis of catecholamines. Its activity is regulated acutely by the phosphorylation of four serines in the N-terminal of the enzyme (1,2). Stimulation of adrenal chromaffin cells with nicotinic agonists or by depolarisation with K^+ causes a Ca^{++}-dependent increase in phosphorylation of three of these serines, Ser^{19}, Ser^{31} and Ser^{40}, and a Ca^{++}-dependent activation of TOH (2,3). The identity of the kinases that mediate the three phosphorylations and which are responsible for the activation of TOH in intact cells is not yet clear.

Ca^{++}/calmodulin-dependent protein kinase II (CAM-K-II) is present in bovine adrenal chromaffin cells and is activated in a Ca^{++}-dependent manner by nicotinic stimulation (4). Its activation correlates with the activation of TOH during nicotinic stimulation (4). *In vitro,* CAM-K-II phosphorylates TOH on Ser^{19} and Ser^{40}, two of the same serines whose phosphorylation is increased by nicotinic agonists and K^+ (2,5) and under appropriate conditions this activates TOH (6). It is therefore possible that CAM-K-II mediates part of the increased phosphorylation and activity of TOH following nicotinic stimulation of chromaffin cells. Two recent studies used newly characterized, selective, membrane-permeant inhibitors of CAM-K-II to investigate its role in the control of catecholamine biosynthesis (7,8). Using rat pheochromocytoma PC12h cells, they found that KN62 inhibited TOH activity stimulated by K^+ depolarisation (9) and KN93 inhibited TOH phosphorylation by both K^+ and acetylcholine and reduced cellular dopamine levels (8). These finding were interpreted as being consistent with a role for CAM-K-II in the acute regulation of TOH activity.

Many protein kinase inhibitors have been found on detailed study to have undesirable nonspecific actions which complicate their use in certain types of experiments. Consequently, we have investigated the ability of the CAM-K-II inhibitors KN62 and KN92 to affect nicotinic activation of TOH in intact bovine adrenal chromaffin cells, and compared this with the actions of structural analogues of these compounds, KN04 and KN92, that are not inhibitors of CAM-K-II (see 10,11).

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MATERIALS AND METHODS

Measurement of TOH activity in intact bovine chromaffin cells. Bovine adrenal chromaffin cells were prepared by collagenase digestion and cultured in 650 ml culture flasks. Cells were used 2–4 days after preparation. TOH activity was measured at 37°C in the intact cells essentially by the method of Meligeni et al. (12) as described in detail by Marley et al. (13). The method measures the production of $^{14}CO_2$ following the hydroxylation and rapid decarboxylation of L-[carboxyl-^{14}C]-tyrosine offered to the cells. Cells were harvested by scraping, washed, equilibrated with buffer, then rewashed and aliquotted into glass tubes. They then received a 15 min preincubation with kinase inhibitors or appropriate vehicle,and then a 10 min incubation with 10 μM ^{14}C-tyrosine (radioactive concentration 2.18 GBq/mmol) in the absence and presence of nicotine or elevated [K^+] (isosmotic substitution for Na^+) in the continued presence of inhibitor or vehicle. During the incubation, the tubes were fitted with a rubber stopper carrying a NaOH trap to absorb emitted $^{14}CO_2$. At the end of the 10 min incubation, cells were lysed with trichloroacetic acid and the tubes left to allow $^{14}CO_2$ to be absorbed. An aliquot of the NaOH was then removed for scintillation counting.

Drugs. KN62, KN04, KN93 and KN92 were from Seikagaku Co, Japan. Nicotine was from Sigma Chemical Co, USA. L-[carboxyl- ^{14}C]-tyrosine (sp. act. 2.18 GBq/mmol) was from Amersham International, UK.

RESULTS

Elevated [K^+] (25 or 50 mM) and nicotine (5 μM) increased TOH activity by 200–600% over 10 min (Figs. 1, 2). These effects were completely dependent on extracellular Ca^{++} (data not shown), as reported previously by others (3,14). The nicotinic activation of TOH was reduced in a concentration-dependent manner by the CAM-K-II inhibitor KN62 (9) (Fig. 1A), with 10 μM KN62 inhibiting the nicotinic response by 76.5 ± 6.3% (mean ± S.E.M., n = 8 cell preparations). KN62 at 10 μM also reduced the TOH activation by K^+ by 71.9 ± 9.6% (mean ± S.E.M., n = 3 cell preparations). In an attempt to confirm that KN62 was inhibiting the K^+ and nicotinic responses by inhibiting CAM-K-II, a structural analogue of KN62 (KN04) that lacks activity as a CAM-K-II inhibitor was also tested (10). KN04 produced a similar concentration-dependent inhibition of nicotine-stimulated TOH activity over the same concentration range as KN62 (Fig. 1B), with 10 μM inhibiting by 77.9 ± 3.6% (mean ± S.E.M., n = 4 cell preparations: $P > 0.1$ compared with the effects of 10 μM KN62 against nicotine or K^+, one-way ANOVA). Both KN62 and KN04 significantly inhibited basal TOH activity in the intact cells (at 10 μM, the inhibitions of basal were 36.3 ± 5.1%, n = 11, and 53.2 ± 4.9%, n = 4, respectively).

An alternative CAM-K-II inhibitor, KN93 (8), was tested to see if it mimicked the actions of KN62. KN93 inhibited TOH activation stimulated by nicotine (Fig 2A) or K^+ (Fig 2C), with an IC_{50} of about 3 μM. Again, a structural analogue of KN93 that lacks activity as a CAM-K-II inhibitor was tested. KN92 (11), although slightly less potent that KN93, produced a similar concentration-dependent inhibition of the activation of TOH by both nicotine and K^+ (Fig 2B,D).

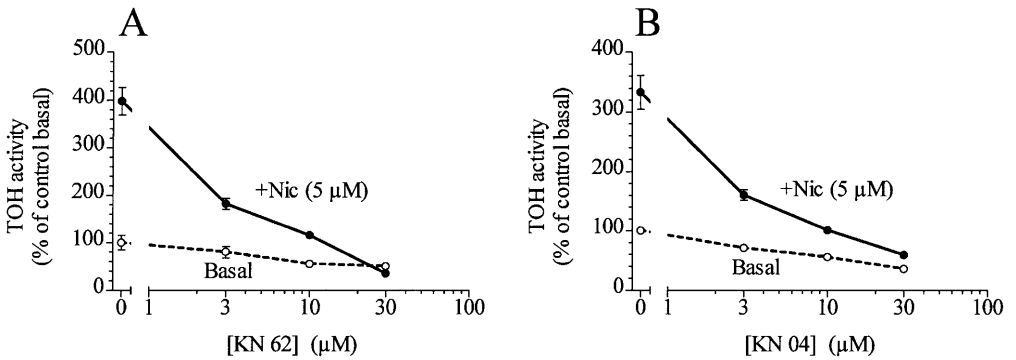

FIG. 1. Effects of (A) KN62 and (B) KN04 on basal (dotted lines) and 5 μM nicotine-stimulated tyrosine hydroxylase activity (solid lines) in intact bovine adrenal chromaffin cells. Data are means ± S.E.M. for n = 4 − 5 from a single preparation of cells and are representative of similar data from 8 cell preparations.

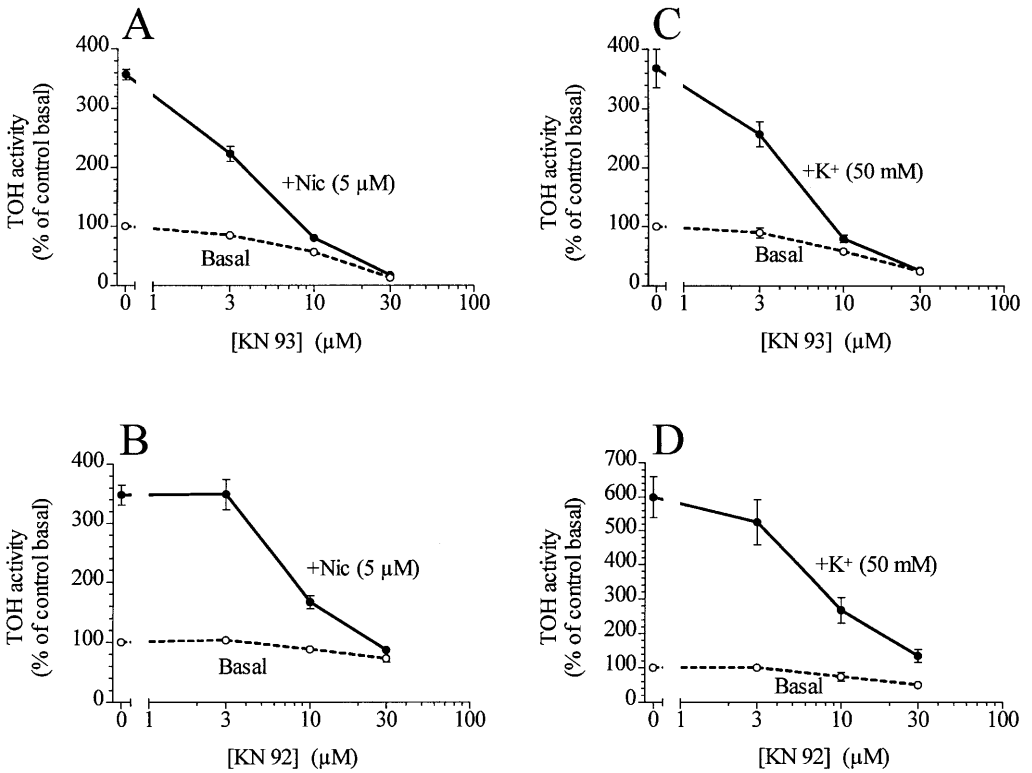

FIG. 2. Effects of (A,C) KN93 and (B,D) KN92 on basal (dotted lines) tyrosine hydroxylase activity and tyrosine hydroxylase activity stimulated by 5 μM nicotine (A,B: solid lines) or 50 mM K^+ (C,D: solid lines). Data are means ± S.E.M. for n = 5 − 6 from a single cell preparation and are representative of data from 3 cell preparations.

DISCUSSION

In studies on rat pheochromocytoma cells, Ishii et al. (9) found KN62 inhibited TOH phosphorylation and the rate of DOPA synthesis *in situ* in PC12h cells. The present results showing that KN62 inhibits K^+ and nicotine-stimulated TOH activity *in situ* in intact bovine chromaffin cells are consistent with these earlier findings. Ishii et al. (9) also found that KN62 had no effect on TOH phosphorylation or DOPA synthesis stimulated by forskolin in PC12h cells, or on the activity of purified TOH *in vitro.* This indicates that KN62 does not inhibit TOH directly, nor does it inhibit the tyrosine transporter in the plasma membrane, which is required to measure *in situ* TOH activity. However in the present study the effect of KN62 on the nicotinic activation of TOH was fully mimicked by KN04, a structural analogue of KN62 that is more than 100-fold less potent as a CAM-K-II inhibitor (10). This suggests that the effects of KN62 on TOH phosphorylation and activity in PC12h cells and bovine chromaffin cells cannot simply be interpreted as being due to an inhibition of CAM-K-II.

The results using KN93 and KN92 further emphasise the need for caution in interpreting the effects of KN62 as being due to CAM-K-II inhibition. KN93 was previously reported to inhibit the acute increases in TOH phosphorylation in rat PC12h cells stimulated by K^+ or acetylcholine, and over several days treatment it reduced cellular levels of dopamine. The present observations that KN93 inhibits TOH activation by K^+ and nicotine *in situ* in intact bovine chromaffin cells is consistent with these previous results. However, the inactive analogue KN92 also inhibited the K^+ and nicotinic responses. The results with KN04 and KN92 strongly suggest that the inhibition of

K^+ and nicotinic activation of TOH by KN62 and KN93 is unlikely to be due to the inhibition of CAM-K-II.

In previous studies using KN62, none of its actions on blood pressure (10), heart rate (10), release of GABA into cerebrospinal fluid (10), numbers of sodium channels in developing skeletal muscle (15), glucose-dependent insulin secretion from β-cells (16), and peptide modulation of synaptic EPSPs (17) were mimicked by KN04. Similarly, in recent studies using KN93, its inhibition of fibroblast cell growth was not mimicked by KN92 (11). Such results suggest that, in a number of different cell types and measuring a number of cellular responses, KN62 and KN93 act as selective and useful membrane-permeant inhibitors of CAM-K-II. The present study suggests that KN62 and KN93 both have important non-specific effects on chromaffin cells that may complicate their use as CAM-K-II inhibitors on these and other cell types, and highlight the need for control experiments with the inactive analogues. A recent study found KN93 had a non-specific protonophoretic action that was not related to CAM-K-II inhibition and was not mimicked by KN62 (18).

The mechanism by which KN62, KN04, KN93 and KN92 all inhibit tyrosine hydroxylase activation is at present not known. Since in both PC12h and bovine chromaffin cells KN62 and KN93 each inhibited TOH activation by K^+, which does not act through nicotinic receptors, these are unlikely to be the site of inhibition of TOH activation. A number of other inhibitors of CAM-K-II have been found to non-specifically block Ca^{++} channels, including W7, W13, calmidazolium and trifluoperazine (19). The present results would be consistent with KN62 and KN93 non-specifically inhibiting voltage-dependent Ca^{++} channels on adrenal chromaffin cells, as well as inhibiting CAM-K-II.

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