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Activation of brain nitric oxide synthase in depolarized human temporal cortex slices: differential role of voltage-sensitive calcium channels

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1 Nitric oxide (NO) synthase activity was studied in slices of human temporal cortex samples obtained in neurosurgery by measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline.

2 Elevation of extracellular K^+ to 20, 35 or 60 mM concentration-dependently augmented L-[^{3}H]-citrulline production. The response to 35 mM KCl was abolished by N^G-nitro-L-arginine (100 μM) demonstrating NO synthase specific conversion of L-arginine to L-citrulline. Increasing extracellular $MgCl_2$ concentration up to 10 mM also prevented the K^+ (35 mM)-induced NO synthase activation, suggesting the absolute requirement of external calcium ions for enzyme activity.

3 However, the effect of high K^+ (35 mM) on citrulline synthesis was insensitive to the antagonists of ionotropic and metabotropic glutamate receptors dizocilpine (MK-801), 6-nitro-7-sulphamoylbenzo(f)-quinoxaline-2-3-dione (NBQX) or L-2-amino-3-phosphonopropionic acid (L-AP3) as well as to the nicotinic receptor antagonist, mecamylamine.

4 The 35 mM K^+ response was insensitive to ω-conotoxin GVIA (1 μM) and nifedipine (100 μM), but could be prevented in part by ω-agatoxin IVA (0.1 and 1 μM). The inhibition caused by 0.1 μM ω-agatoxin IVA ($\sim$30%) was enhanced by adding ω-conotoxin GVIA (1 μM) or nifedipine (100 μM). Further inhibition (up to above 70%) could be observed when the three Ca^{2+} channel blockers were added together. Similarly, synthetic FTX 3.3 arginine polyamine (sFTX) prevented (50% at 100 μM) the K^+-evoked NO synthase activation. This effect of sFTX was further enhanced (up to 70%) by adding 1 μM ω-conotoxin GVIA plus 100 μM nifedipine. No further inhibition could be observed upon addition of MK-801 or/and NBQX.

5 It was concluded that elevation of extracellular [K^+] causes NO synthase activation by external Ca^+ entering cells mainly through channels of the P/Q-type. Other Ca^{2+} channels (L- and N-type) appear to contribute when P/Q-channels are blocked.

Keywords: NO synthase; human brain; Ca^{2+} channels; ω-agatoxin IVA; ω-conotoxin GVIA; nifedipine; sFTX; glutamate receptors; nicotinic receptors

Introduction

Nitric oxide (NO) is formed from L-arginine under the catalysis of NO synthase (Knowles *et al*., 1989). Brain localization of NO synthase immunoreactivity suggesting a neuromodulatory role for NO has been demonstrated by several authors in a number of mammalian species. The regional distribution of NO synthase in the human brain has recently been described (Egberongbe *et al*., 1994).

It is known from animal studies that NO is produced in response to an increase in intracellular Ca^{2+} ions (Garthwaite *et al*., 1988; East & Garthwaite, 1991). In fact, constitutive NO synthase of rat brain has an absolute requirement for Ca^{2+}/calmodulin (Bredt & Snyder, 1989; Forstermann *et al*., 1991). Calcium ions able to stimulate NO synthase may originate from the extracellular space through voltage-dependent Ca^{2+} channels (Alagarsamy *et al*., 1994a; Lizasoain *et al*., 1995), ionotropic glutamate receptors (Bredt & Snyder, 1989; East & Garthwaite, 1991) and from intracellular Ca^{2+} stores. In addition, it has been shown that neuronal nicotinic receptor activation can lead to calcium influx into neuronal cells (Fieber & Adams, 1991; Sands & Barish, 1991; Vernino *et al*., 1992; Barrantes *et al*., 1995).

Exposure of brain tissue to high K^+ media is one condition expected to cause opening of voltage-dependent Ca^{2+} channels; K^+-depolarization is also capable of inducing the release of glutamate/aspartate onto ionotropic and metabotropic glutamate receptors or of acetylcholine onto nicotinic receptors and therefore to increase the availability of intracellular Ca^{2+} ions to calcium acceptors. Investigating the regulation of the enzyme responsible for NO production may provide insight into the process in which the gaseous messenger has been implicated. It should be added that elevations of extracellular [K^+] can occur in a number of pathological conditions such as cerebral ischaemia (Croning *et al*., 1995; Zetterström *et al*., 1995), spreading depression (Hansen & Zeuthen, 1981; Kraig *et al*., 1991) and epilepsy (Somjen, 1979; Louvel *et al*., 1994).

Human brain NO synthase has so far not been characterized in experiments of functional neurochemistry. In this work slices from human temporal cortex specimens obtained during neurosurgery were utilized to study the effect of K^+-depolarization on the activity of NO synthase and to investigate how Ca^{2+} ions gain access to the intracellular enzyme.

Methods

Fresh human brain cortex specimens were obtained from patients undergoing neurosurgery to reach deeply located tumours (2) or from patients suffering untreatable temporal lobe epilepsy (10); each experiment was performed from the tissue of a single patient within 2 h from surgery. Since we did not observe significant differences between results obtained from the two groups of patients, all data have been pooled. Slices of temporal cortex from 12 patients (10 males and 2 female; aged 25–60 years) were obtained by means of a McIlwain tissue chopper set at 400 μm and equilibrated for 1 h at 37°C in a

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medium having the following composition (mM): NaCl 145, KCl 3, $CaCl_2$ 0.2, $MgSO_4$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 1, glucose 10, gassed with 95% O_2 and 5% CO_2, pH 7.2–7.4. Nitric oxide synthase activity was measured essentially according to Alagarsamy *et al.* (1994a) with minor modifications. Briefly, each slice was transferred in 2 ml of warm medium and preincubated in the presence or absence of drugs for 20 min. Preincubation medium was removed and slices were then depolarized for 5 min, in the presence or absence of drugs, with 2 ml of medium containing KCl 35 mM (replacing an equiosmolar amount of NaCl) and supplemented with 30 nM [^{3}H]-arginine and 970 nM arginine. The KCl concentration was chosen on the basis of preliminary pilot experiments in which slices were depolarized with medium containing 20, 35 or 60 mM KCl (replacing the respective equiosmolar amounts of NaCl). Control slices (medium with 3 mM KCl) were always run in parallel. At the end of the incubation, the reaction was stopped, slices were washed with 3×5 ml of ice-cold medium in the presence of 3 mM arginine, 4 mM citrulline and 4 mM EDTA and then transferred into acetic acid (1 N; 90°C). After 10 min, slices were sonicated and centrifuged at $12000 \times g$ for 15 min; the supernatant was freeze-dried and reconstituted in HEPES buffer, pH 7. [^{3}H]-citrulline (eluted at pH 7 with HEPES buffer) was resolved from [^{3}H]-arginine (eluted at pH 14 with NaOH 1 N) by means of an AG 50W-X4 (Bio Rad) column. Unspecific conversion, which amounted to approx. 4%, was determined on boiled tissue slices and subtracted from each data point. Radioactivity was determined by liquid scintillation counting. Data have been expressed as the amount of [^{3}H]-citrulline divided by the amount of [^{3}H]-citrulline + [^{3}H]-arginine $\times 100$ and analysed by ANOVA followed by Dunnett's test or Duncan multiple comparison tests when appropriate. Effects were considered significant at the level of $P<0.05$.

Chemicals

[^{3}H]-arginine monohydrochloride (specific activity, 60 Ci $mmol^{-1}$) was purchased from Amersham Radiochemical Centre (Buckinghamshire, U.K.); L-arginine hydrochloride, L-citrulline, nifedipine and mecamylamine from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ω-conotoxin GVIA from Peninsula Laboratories Europe (St. Helens, Merseyside, U.K.); L-2-amino-3-phosphonopropionic acid (L-AP3) and N^G-nitro-L-arginine (L-NOARG) from Tocris Cookson (Bristol, U.K.). The following drugs were generous gifts from the companies indicated in parentheses: 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(f)quinoxaline (NBQX; Nova Nordisk, Malov, Denmark); dizocilpine (MK-801; Merck Sharp & Dohme, Harlow, Essex, U.K.); ω-agatoxin IVA (Pfizer Central Research Division, Groton, CT, U.S.A.); sFTX (Lilly Research Centre Limited, Windlesham, Surrey, U.K.). NBQX (10 mM) and nifedipine (100 mM) were dissolved in dimethyl sulphoxide (DMSO) and diluted to the final concentrations in medium; the equivalent amount of DMSO was always added to the corresponding controls.

Results

Exposure of human temporal cortex slices to high-K^+ media elicited a concentration-dependent enhancement of [^{3}H]-citrulline formation from [^{3}H]-arginine with a 500% increase reached at 60 mM (Figure 1). The effect of 35 mM KCl depolarization was completely blocked by the NO synthase inhibitor L-NOARG (100 μM) or by 10 mM $MgCl_2$ (Figure 2). However, under unstimulated conditions, the basal conversion, which amounted to $3.98 \pm 0.22\%$ ($n=32$ slices), was not affected by either L-NOARG or $MgCl_2$.

As shown in Table 1, the 35 mM K^+-evoked stimulation of NO synthase was insensitive to 30 μM dizocilpine (MK-801), a blocker of the NMDA receptor channel, to 50 μM 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2-3-dione (NBQX), an an-

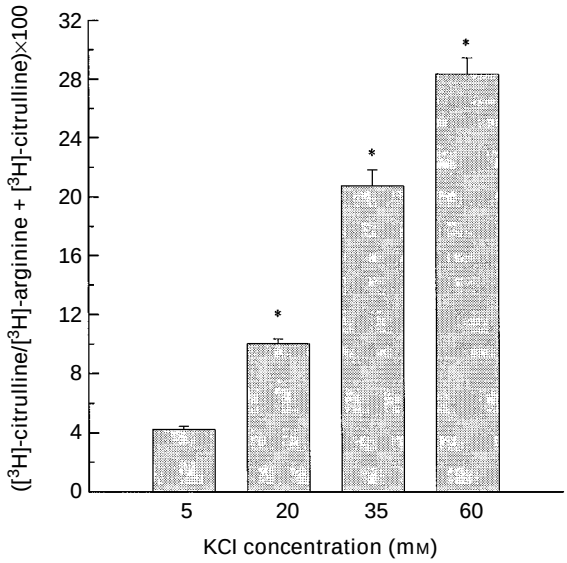

Figure 1 Depolarization-induced activation of NO synthase in human temporal cortex slices: effects of different KCl concentrations. Human cortex slices (400 μm thickness) were equilibrated for 1 h at 37°C in standard medium gassed with 95% O_2 and 5% CO_2, pH 7.4; NO-synthase activity was assessed by incubating the tissues for 5 min in medium containing 3 (controls), 20, 35 or 60 mM KCl (replacing the respective equiosmolar amount of NaCl) supplied with a radiolabelled mixture of arginine 1 μM. Data are expressed as the ratio between labelled citrulline and total radioactivity present in the slice (citrulline + arginine) and represent mean ± s.e.mean of 8 slices from at least 3 different experiments. For further technical details see Methods. $^*P<0.05$ when compared to 3 mM KCl (controls).

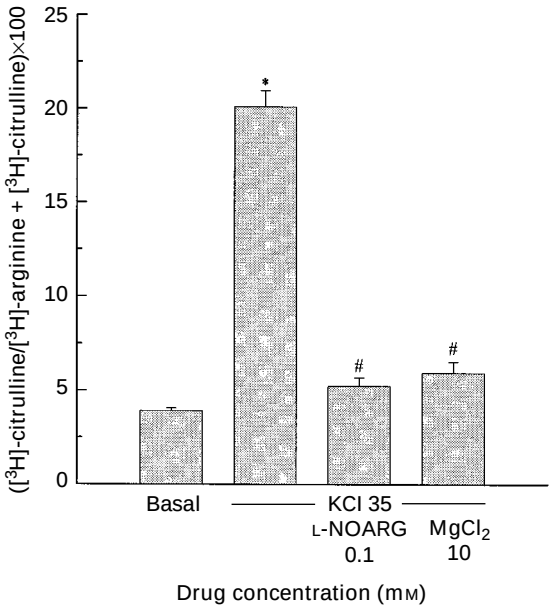

Figure 2 Effect of KCl-induced depolarization on NO synthase activity in human temporal cortex slices: sensitivity to L-NOARG and to Ca^{2+} ions. Human cortex slices were equilibrated for 1 h at 37°C in standard medium gassed with 95% O_2 and 5% CO_2 pH 7.4 and then preincubated for 20 min (2 ml medium) in the presence or absence of drugs. Depolarization of the tissue was carried out by exposing the slices to medium (2 ml) containing KCl 35 mM for 5 min in the presence of a radiolabelled mixture of arginine 1 μM. Some slices (control) were not exposed to high KCl to evaluate the production of citrulline under basal conditions. In the treated slices, L-NOARG (100 μM) or $MgCl_2$ (10 mM) were present during the depolarization. Data are expressed as the ratio between labelled citrulline and total radioactivity present in the slice (citrulline + arginine) and represent mean ± s.e.mean of 6–32 slices from at least 3 different experiments. For further technical details see Methods. $^*P<0.05$ when compared to basal; $\#P<0.05$ when compared to KCl 35 mM.

tagonist at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors and to 300 μM L-2-amino-3-phosphonopropionic acid (L-AP3), an antagonist at metabotropic glutamate receptors. The KCl-induced increase was not affected even when MK-801 (30 μM) and NBQX (50 μM) were present together. Mecamylamine, a selective antagonist at the nicotinic cholinoceptor, was also ineffective in altering the depolarization-evoked production of [^{3}H]-citrulline.

Figure 3 illustrates the effects of various Ca^{2+} channel antagonists on the K^+-induced activation of NO synthase. The K^+ response was insensitive to the N-type blocker ω-conotoxin GVIA (1 μM; Olivera *et al*., 1985; Mogul & Fox, 1991; Regan *et al*., 1991) or to the L-type antagonist nifedipine (100 μM; Hofmann *et al*., 1994). Significant inhibition (~30%) was produced by 0.1 μM of the P/Q-type channel blocker ω-agatoxin IVA (Mintz *et al*., 1992) which, at 1 μM, blocked the NO synthase stimulation by about 50%.

Mixtures of ω-agatoxin IVA (0.1 μM) and nifedipine (100 μM) or ω-conotoxin GVIA (1 μM) produced inhibitions of the K^+-evoked NO synthase elevation significantly more pronounced than that of 0.1 μM ω-agatoxin IVA alone. Figure 3 also shows that the NO synthase response was further inhibited (up to 70%) when the slices were exposed to a combination of ω-agatoxin IVA (0.1 μM), nifedipine (100 μM) and ω-conotoxin GVIA (1 μM).

The NO synthase activation elicited by high-K^+ was also inhibited by sFTX, a synthetic polyamine shown to block specifically Ca^{2+} channels of the P/Q-type (Dupere *et al*., 1996). Addition of 100 μM sFTX reduced the NO synthase response by 50% and the response was practically abolished when slices were exposed to 1 mM of the synthetic polyamine (Figure 4). The figure also shows that potentiation of the sFTX (100 μM) effect (up to 70%) could be obtained by adding to the polyamine nifedipine (100 μM) and ω-conotoxin GVIA (1 μM). On the other hand, the presence of the glutamate receptor antagonists MK-801 or/and NBQX, together with the three Ca^{2+} channel blockers in combination, did not inhibit further the NO synthase response, about 30% of which remained unaffected.

Table 1 Effects of glutamate and nicotine receptor antagonists on the KCl-induced activation of NO synthase in human temporal cortex slices

Drugs (μM)	*KCl-induced NO synthase activation* ($[^3H]$-citrulline/($[^3H]$-arginine + $[^3H]$-citrulline) × 100
Control	15.835 ± 0.982 (30)
MK-801 (30)	13.405 ± 1.063 (15)
NBQX (50)	13.880 ± 0.761 (8)
L-AP3 (300)	13.874 ± 0.658 (6)
MK-801 (30) plus NBQX (50)	15.557 ± 1.020 (8)
Mecamylamine	16.256 ± 1.924 (8)

Human temporal cortex slices were equilibrated for 1 h at 37°C in standard medium continuously gassed with O_2:CO_2 95:5%, pH 7.4. Drugs were present 20 min before and together with the 5 min KCl (35 mM) pulse. Net production has been calculated by subtracting the basal production of citrulline from that measured under depolarizing conditions. Data represent mean ± s.e.mean of (*n*) slices from at least 3 different experiments. For further technical details see Methods.

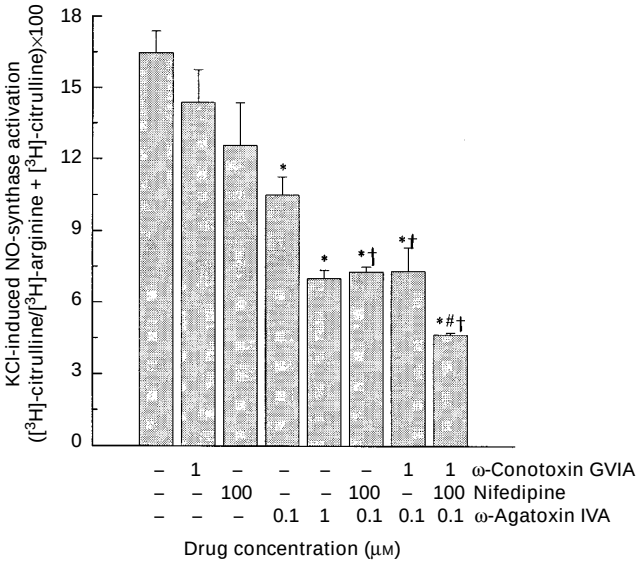

Figure 3 Effects of different Ca^{2+} channel antagonists on the KCl-induced activation of NO synthase in human temporal cortex slices. Results, presented as the ratio between labelled citrulline and total radioactivity present in the slice (citrulline + arginine), represent the net production calculated by subtracting the production of citrulline measured under unstimulated conditions from that elicited by 35 mM KCl depolarization. Data represent the mean ± s.e.mean of 6–32 slices from at least three different experiments. See also legend to Figure 1 and Methods. *$P<0.05$ versus controls; †$P<0.05$ versus ω-agatoxin 0.1 μM; #$P<0.05$ versus ω-agatoxin 0.1 μM + nifedipine 100 μM or ω-agatoxin 0.1 μM + ω-conotoxin 1 μM.

Discussion

Depolarization of human temporal cortex slices with high-K^+ enhanced the rate of conversion of [^{3}H]-arginine to [^{3}H]-citrulline. Since this effect was prevented by L-NOARG and by raising extracellular Mg^{2+} concentration, K^+-depolarization is likely to cause activation of constitutive Ca^{2+}-dependent NO synthase by facilitating Ca^{2+} influx into the structures containing the enzyme. However, under our experimental conditions, the basal rate of conversion of L-arginine into L-citrulline was not affected by L-NOARG or $MgCl_2$ at concentrations which completely prevented the K^+ effect, thus suggesting its non NO synthase origin, although a contribution of L-NOARG-insensitive NO synthase isoforms cannot be excluded (Lambert *et al*., 1991; Moncada & Higgs, 1991). It should be also added that the basal conversion of arginine into citrulline in the tissue from epileptic patients might be altered

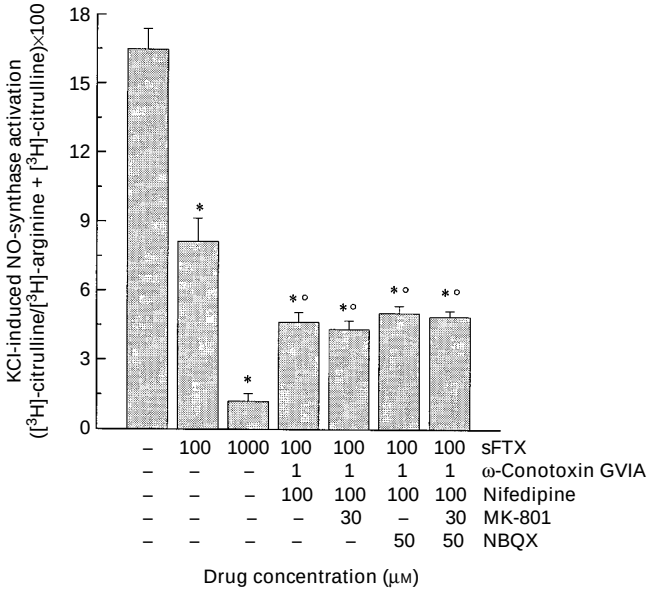

Figure 4 Effects of various Ca^{2+} channel and glutamate receptor antagonists on the KCl-evoked activation of NO synthase from human temporal cortex slices. Results are expressed as in Figure 3. Data represent the mean ± s.e.mean of 6–32 slices from at least three different experiments. *$P<0.05$ versus controls; °$P<0.05$ versus sFTX 100 μM.

by the pathology and therefore it might turn out that resting efflux could be lower in 'normal' tissue. In the human cerebral cortex, most of the cells immunopositive for the cytosolic NO synthase have been identified as bipolar and multipolar non-pyramidal neurones (Egberongbe *et al*., 1994). Calcium ions could penetrate into these cells during activation of voltage-sensitive Ca^{2+} channels by high K^+ and of Ca^{2+}-permeant ionotropic glutamate and/or nicotinic receptor channels by the respective endogenous neurotransmitters released during depolarization.

Multiple Ca^{2+} channels may exist on the NO synthase-containing cells. The findings that ω-agatoxin IVA and sFTX could prevent in part the NO synthase response elicited by high-K^+, whereas neither ω-conotoxin GVIA nor nifedipine could significantly affect, on their own, the production of NO suggest that P/Q-channels are major contributors to the activation of the intracellular Ca^{2+} acceptor. Based on experiments with FTX, involvement of P-channels had previously been proposed in the NO synthase elevation provoked by high-K^+ in slices of rat neocortex (Alagarsamy *et al*., 1994a). Interestingly, when P/Q-type channels in human brain slices were blocked by 0.1 μM ω-agatoxin IVA, addition of the N-type blocker ω-conotoxin GVIA or of the L-channel antagonist nifedipine potentiated the inhibition of the NO synthase response; this inhibition was further strengthened when the three blockers were present together.

That different Ca^{2+} channel antagonists can co-operate in a synergistic/additive manner to prevent the elevation of NO synthase activity provoked by K^+-depolarization may have different explanataions. One possibility is that N- and L-type channels are recruited when NO synthase is no longer saturated by the Ca^{2+} ions provided by the P/Q-channels. Our data are compatible with the view that P/Q-, N- and L-channels coexist on the same NO synthase-containing structure.

Similar effects could also be observed when Ca^{2+} channel blockers *per se* ineffective were combined with relatively low concentrations of sFTX, a synthetic polyamine shown to block P/Q-channels in brain slices (Dupere *et al*., 1996). It has to be noted that the NO synthase response to high-K^+ was completely blocked by sFTX alone, when the drug was added at 1 mM. Clearly, the data showing the effects of antagonist mixtures could not be easily explained if one assumes that sFTX is a selective blocker of the P/Q-type Ca^{2+} channel also at 1 mM. In fact, non-selective current blockade by 1 mM sFTX has been suggested to occur in electrophysiological studies of Ca^{2+} channels in rat neocortical neurones (Brown *et al*., 1994; Pearson *et al*., 1995; Norris *et al*., 1996). If this is also the case in our system, the results illustrated in Figures 3 and 4 indicate that a component of the NO synthase response ($\sim$30%), apparently dependent on extracellular Ca^{2+}, remains insensitive to P/Q-, N- and L-channel blockers, although it could be practically abolished by high (1 mM) non-selective concentrations of sFTX (Figure 4).

Depolarization of brain slices with high-K^+ is known to elicit release of glutamate/aspartate. It was shown that the K^+-evoked release of glutamate from synaptosomes involves activation of presynaptic Ca^{2+} channels of the P-type (Turner *et al*., 1992). The excitatory amino acids released by depolarization are expected to bind to glutamate receptors (NMDA or/and AMPA/kainate) mediating intracellular penetration of Ca^{2+} ions and, possibly, NO synthase activation. The lack of effect of MK-801, a blocker of the Ca^{2+} channel of NMDA receptors, and of NBQX, a competitive antagonist at AMPA/kainate receptors, alone or in combination excludes a major involvement of the above pathway in the K^+-evoked NO synthase response. In particular, the P/Q-channels activated by high-K^+ may not be presynaptic on glutamatergic terminals, but are probably situated on the NO synthase-containing cells. Second, activation of NMDA or AMPA/kainate receptors play no significant role in the NO synthase response elicited by K^+ depolarization of human neocortical slices. Similar conclusions had been reached in experiments with rat neocortex slices depolarized with high K^+ (Alagarsamy *et al*., 1994a). In apparent contrast, the NO synthase activation caused by veratrine in rat forebrain slices was shown to be partially blocked by MK-801 (but not by NBQX) and by ω-conotoxin GVIA (Lizasoain *et al*., 1995). However, addition to rat brain slices of exogenous NMDA (in Mg^{2+}-free medium), but not AMPA, increased the activity of the enzyme (Alagarsamy *et al*., 1994b).

Moreover, the involvement of L-AP3-sensitive metabotropic glutamate receptors, the activation of which would lead to increases of cytosolic Ca^{2+} by releasing it from inositol-1, 4, 5-triphosphate-sensitive stores, can be excluded since the addition of the selective antagonist did not decrease significantly the K^+-evoked NO synthase activation (Table 1).

In addition to glutamate receptors, it has been recently shown that activation of neuronal nicotinic cholinoceptors leads to Ca^{2+} ions influx (Fieber & Adams, 1991; Sands & Barish, 1991; Vernino *et al*., 1992; Barrantes *et al*., 1995; Gray *et al*., 1996) which, in principle, might stimulate NOS activity. However, these cholinoceptors do not seem to play a significant role in our model since the KCl-induced citrulline formation was not affected by the selective nicotinic receptor antagonist mecamylamine. Nevertheless, the involvement of transmitters other than glutamate and acetylcholine released from P/Q-channel bearing nerve terminals cannot be entirely excluded.

To conclude, increases of extracellular [K^+] in human brain can lead to elevation of NO production. This occurs because extracellular Ca^{2+} enters into NO synthase-containing cells through voltage-sensitive channels. Penetration of Ca^{2+} through ionotropic glutamate and nicotinic receptors or mobilization of internal Ca^{2+} by L-AP3-sensitive metabotropic glutamate receptor activation appears to play no significant role. Calcium channels of the P/Q-type contribute most to the NO synthase response to high-K^+, although N- and L-channels may co-operate with P/Q-channels. Assuming that the present scenario reflects what occurs in some of the physiopathological conditions in which NO has been implicated, association of Ca^{2+} channel antagonists or non-selective blockers may deserve better consideration than selective P/Q-channel blockers as potential therapeutic agents.

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