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EFFECTS OF GLUTAMATE AND ZINC IONS ON THE CONTRACTILITY OF VASCULAR SMOOTH MUSCLE PREPARATIONS

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Abstract

It was shown in this study that isolated porcine coronary arteries (PCA) contracted by depolarization with high K_o or by histamine are dose-dependently relaxed by glutamic acid, aspartic acid, N-methyl-asparate (NMDA) and γ-aminobutyric acid (GABA). Zn^{2+} was also shown to relax dose-dependently PCA contractions induced by 50 mM KCl with an ED_{50} value of about 1.5 mM and to inhibit dose-dependently histamine-induced contractions, shifting ED_{50} values from 6μM to 40 μM, not affecting however corresponding cumulative concentration-response (CCR) curves established for acetylcholine-induced contractions. Furthermore, since Zn^{2+} ions are co-localized in many glutamatergic synapses of the central nervous system, it has been postulated in analogy to glutamate neurotoxicity that perturbations of the synaptic zinc concentrations might be a triggering factor in several cerebral diseases, such as ischemic strokes and sustained seizures. Unfortunately, little is known so far about effects of glutamate and zinc ions on the vascular tone. Although the nature of the glutamatergic receptors occurring in the blood vessels investigated in this study remains unclear, the results suggest that glutamate and Zn^{2+} ions interact with voltage-gated as well with ligand-operated Ca-channels. An interesting aspect might be the putative role of glutamate and zinc as long-term toxic agents in the early steps of the pathomechanisms leading to degenerative vascular lesions.

Key words: monosodium glutamate, aspartate, N-methyl-D-aspartate (NMDA), GABA, zinc ions, blood vessels.

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INTRODUCTION

Glutamate receptors, exhaustively investigated in the central nervous system (CNS), are classified according to the nature of the signal transduction mechanism, with ionotropic receptors acting as ligand-gated cation channels and metabotropic receptors operating via second messengers (DINGLEDINE et. al. 1999, DANYSZ, PARSON 1998). Ionotropic receptors gated by L-glutamate are distinguished on the basis of pharmacological criteria into three main subtypes: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5--methylisoxazole-4-propionic acid (AMPA) and kainate receptors. NMDA receptors are characterized by high Ca permeability and a voltage-dependent Mg^{2+} block. The gating of the associated ion channels is modulated by glycine (acting as a co-agonist to glutamate) and by Zn^{2+} ions. Zn^{2+} ions are also present in synaptic glutamatergic vesicles and co-released with glutamate. NMDA receptors have been shown to play an important role in several cerebral functions endowed with high synaptic plasticity, such as learning and memory; however, they have also been implicated in neuronal injury and death caused by massive release of L-glutamate, which might take place in several types of traumatic lesions, such as a head injury, stroke, epilepsy and chronic neurodegenerative diseases (BENVENISTE et. al. 1984, FADEN et al. 1989, BARNES 1988, OLNEY 1990). Quite similar neurotoxicity has been postulated for zinc ions (CHOI, KOH 1998). Notwithstanding the ambiguous role played by glutamate and zinc in the cerebral neurotransmission, both substances are widely present in daily life, with a reputation of being "virtually nontoxic" (CHOI, KOH 1998, VALEE, FALCHUK 1993). The aims of the present study were: (a) to investigate the effects of glutamate and other related substances with transmitter function and of Zn^{2+} ions on the contractility of arterial preparations activated by different stimuli and (b) to detect a putative deleterious effect of excess glutamate on the vascular cells similar to the excitotoxicity observed in neurons, whereby a possible implication as triggering factors in pathological lesions of the vascular wall might also be discussed.

MATERIAL AND METHODS

Large branches of epicardial coronary arteries (right, anterior descending and circumflex arteries; outside diameter 2.5 mm-0.9 mm) were prepared from pig hearts freshly obtained from a nearby slaughterhouse. The vessel preparations were cut into helical strips (approx. 10-15 mm in length and 2 mm in width), pierced by two hooks and mounted vertically in 6 parallel setups of organ baths each filled with 25 ml of physiological salt solution (PSS) maintained at 37°C and aerated continuously with 95% O_2 and 5% CO_2. The PSS had the following composition (mM): NaCl 137; KCl 2.7;

$CaCl_2$ 1.4; $MgCl_2$ 0.5; $NaHCO_3$ 11.9; NaH_2PO_4 0.4; Glucose 8. The changes in force or length developed by the preparations were measured isometrically (Statham Instruments transducers) or isotonically (HF-Modem-Hugo Sachs Elektronik K.G.) and registered on conventional chart recorders. The maximal wall stress (force/cross sectional area; mN mm^{-2}) was approximated by multiplying the force by the tissue length and dividing by the wet weight. The following chemicals were used: aspartic acid, α-aminobutyric acid (GABA), 3.4-diaminopyridine (3.4-AP), glutamic acid, glycine, histamine dihydrochloride, NaF, tetraethylammonium (TEA), $ZnCl_2$. All the drugs were purchased from Sigma. The data were expressed as the mean ± S.E.M. and the number of strip preparations excised from the same coronary artery. For statistical analysis, Student's t-test for paired and unpaired values was used. P values less than 0.05 were considered significant.

RESULTS

1. Relaxations induced by glutamate, GABA, NMDA and aspartate in PCA depolarized by high K or stimulated by histamine

Whereas the basal tone of unstimulated PCA preparations was not affected by application of glutamate or related agonists (GABA, NMDA, aspartate), preparations contracted by depolarization with high K_o or after stimulation with 10 mM histamine were strongly relaxed by glutamate (0.1-5 mM). However, the relaxing efficacy differed according to the type of activation. The amplitude of the relaxation induced by glutamate (5 mM) in preparations depolarized by high external K^+ and contracted via Ca influx through L-type Ca channels, was much smaller than that recorded in histamine--stimulated preparations (Table 1).

Table 1

Comparison of the magnitude of relaxations induced by glutamate, aspartate, NMDA and GABA on PCA preparations contracted by KCl (50 mM) and by histamine (10 μM). The values are expressed as percentage of the maximal height of contraction obtained by depolarization with 50 mM KCl and of the tonic component of the biphasic contraction induced by 10 μM histamine, respectively. 100 % correspond to the values in mN mm^{-2} given in the parentheses; n = 6 for each agonist

	KCl 50 mM	Histamine 10 μM
Glutamate 5 mM	18.1 ± 4.3% (14.9 ± 1.3 mN mm^{-2})	92.5 ± 3.1% (9.1 ± 1.3 mN mm^{-2})
Aspartate 5 mM	-	78.8 ± 8.8 % (1.5 ± 0.5 mN mm^{-2})
NMDA 1.5 mM	10.2 ± 1.6% (13.8 ± 1.5 mN mm^{-2})	34.0 ± 6.8% (3.9 ± 0.6 mN mm^{-2})
GABA 5 mM	19.8 ± 3.2% (14.9 ± 1.8 mN mm^{-2})	48.3 ± 4.9% (2.9 ± 0.5 mN mm^{-2})

Moreover, in the first case the relaxations were transient, whereas in histamine-stimulated preparations the relaxations were sustained. Contrasting with the general observation that histamine characteristically exerts a predominantly dilator effect on vascular smooth muscle, very powerful contractions were recorded when histamine was applied on PCA preparations at all concentrations (Figures 1 and 2); these contractions are most probably linked to the activation of ligand-operated receptor channels. Figure 1 summarizes the relaxing effect of several agonists of glutamatergic receptors on PCA preparations stimulated by 50 mM KCl or by 10 μM histamine. The largest relaxations were those induced by aspartate, NMDA and GABA on preparations stimulated by histamine.

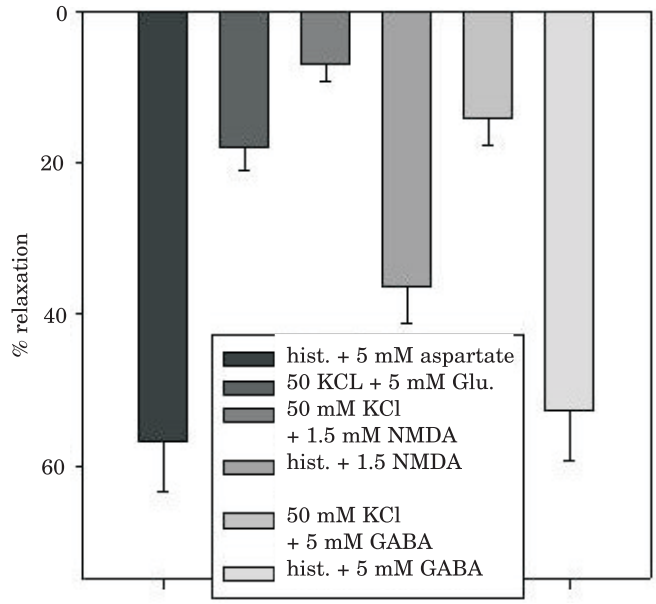

Fig. 1. Effects of NMDA-agonists on PCA stimulated by 50 mM KCl or by 10 μM histamine. The error bars represent means values ± SEM and n = 6 for each column. See text for further details

At first sight, the finding that glutamate as well as the other investigated agonists relax contractions induced by histamine or by depolarization with high K^+ is unexpected, since it is well-known that in the CNS glutamate acts mainly as an excitatory neurotransmitter. If gating of the channels associated with glutamatergic receptors results in Ca entry, then contractions would be expected to occur when glutamate is added to stimulated PCA preparations. The results presented in Figure 1 show that the contrary is observed and that without any exception only relaxations are recorded. Although no experimental evidence is presented here to support the following

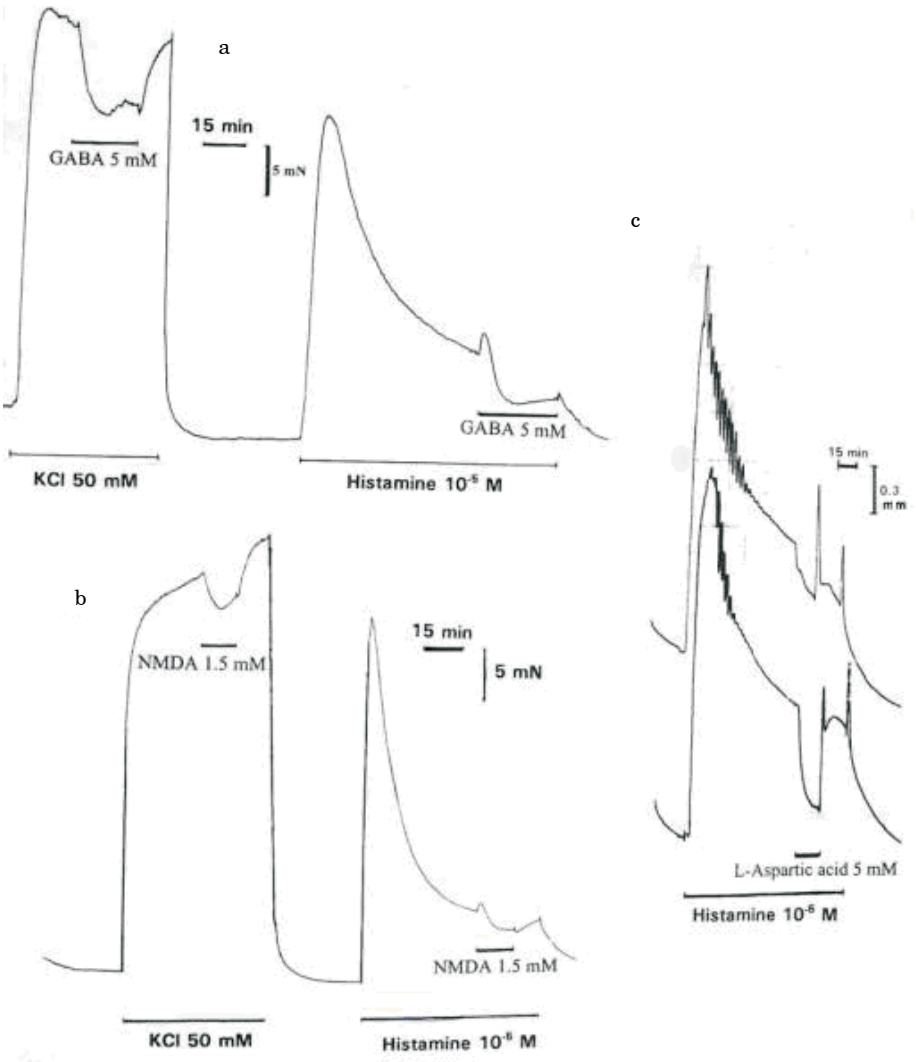

Fig. 2. Representative mechanograms for the relaxations induced by GABA (a), for NMDA (b) and for aspartic acid (c)

assumption, a rational explanation for the contradiction encountered in this study may be that the stimulation of glutamatergic receptors in the vascular smooth muscle, which has not been investigated until now, is associated with an increasing potassium conductance. This point will be further explained in details in the discussion. Figure 2 shows representative experiments obtained for GABA (a), NMDA (b) and aspartate (c).

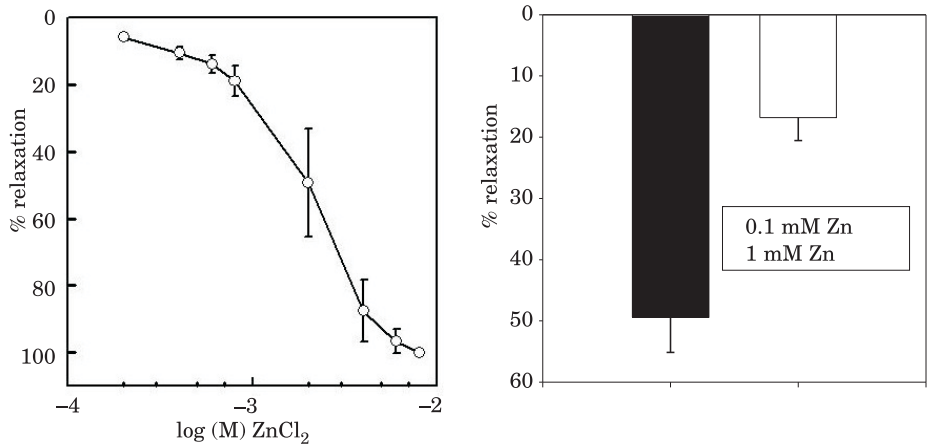

Fig. 3. The diagram on the left shows the relaxations induced by cumulative addition of zinc ions on PCA depolarized by high K^+. 100 % correspond to the maximal contractions induced by 50 mM KCl: 17 mN mm^{-2}; $n = 6$. The diagram on the right shows the effects of two zinc ions concentrations (0.1 and 1 mM) on the relaxations induced by 5 mM glutamate on PCA preparations contracted by 10 μM histamine. The error bars represent means values ± SEM

2. Inhibiting effects of Zn^{2+} ions on depolarization-induced PCA-contractions and on relaxations induced by glutamate in histamine-stimulated preparations

On the left side of Figure 3 it is shown that Zn^{2+} ions relax dose-dependently PCA contractions induced by 50 mM external K^+ with an ED_{50} value of approx. 1.5 mM. This finding is easily interpreted, if it is assumed that the depolarization induced by high external K^+ opens a Ca-channel of L-type, leading to the contraction of the vascular smooth cells. Thus Zn^{2+} ions seem able to inhibit Ca-entry by binding to a specific site of the channel pore.

On the right side of Figure 3 it can be seen that addition of Zn^{2+} ions reduces the amplitude of the relaxations induced by 5 mM glutamate in histamine-stimulated PCA preparations. They amount to resp. 49.38 ± 5.77% with 0.1 mM Zn^{2+} and to 16.82 ± 3.72% with 1 mM Zn^{2+}; $n = 12$; $p < 0.05$. The diagram on the left side of the same figure shows relaxations induced by cumulative addition of zinc ions on PCA depolarized by high K^+. 100% correspond to the maximal contractions induced by 50 mM K^+: 17 mN mm^{-2}; $n = 6$. The results presented in Figure 3 are consistent with the ability of Zn^{2+} ions to act as a modulator of both voltage-sensitive and ligand-gated ion channels. Thus, taken together the results seem to suggest that Zn^{2+}

ions modify the kinetics of ion channels by binding to sites located on Ca or K channels.

3. Effects of Zinc ions on PCA contractions induced by histamine and by acetylcholine

Figure 4 shows the effects of $ZnCl_2$ (0.5 mM) on contractions induced by histamine and by ACh. Whereas CCR-curves for histamine-induced contractions were significantly shifted to the right (ED50 values increased from 0.9 µM to 4 µM), indicating a blockade of Ca entry through ligand-gated channels by Zn^{2+} ions, the same treatment did not affect the corresponding effects induced by ACh.

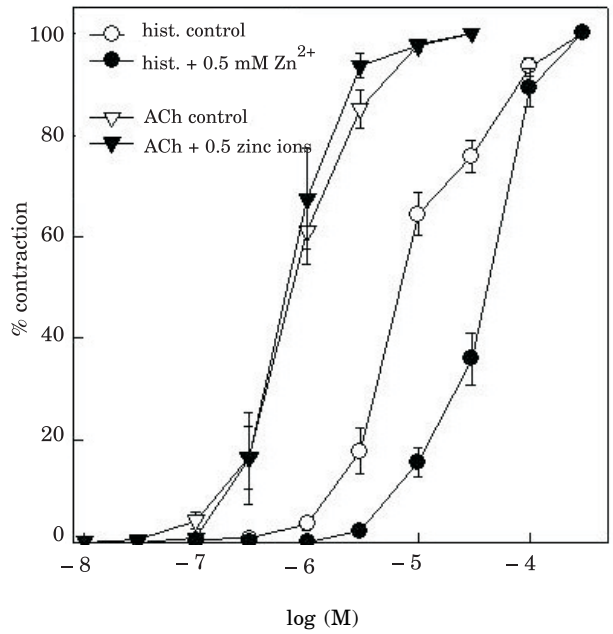

Fig. 4. Effects of $ZnCl_2$ (0.5 mM) on contractions induced by histamine and ACh (n = 12). 100 % =9.98 ± 1.2 for histamine and 8.1 ± 1.5 mN mm^{-2} for ACh, n = 12. The error bars represent mean values ± SEM

4. Effects of K channel blockers on glutamate-induced relaxations of PCA stimulated by histamine

In order to test the possibility that glutamate-induced relaxations are caused by an increased potassium conductance, PCA preparations relaxed by glutamate during a stimulation with histamine were treated with K-channel blockers (3, 4-diaminopyridine and tetraethylammonium). As can be seen in Figure 5 (third and fourth column from the left) application of 1 mM 3, 4-diaminopyridine reversed completely the relaxations induced by glutamate in the histamine-stimulated preparations. A similar treatment of the preparations with 5 mM TEA was not able to significantly affect the glutamate-induced relaxations. The amplitude of contractions induced by 50 mM K^+ and 10 μM histamine are shown in the first and second column of Figure 5 in order to allow a comparison with the glutamate-induced effects.

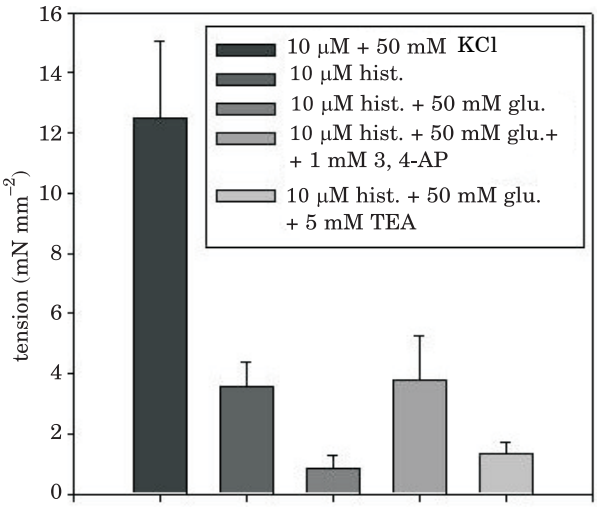

Fig. 5. Effects of K channel blockers on glutamate-induced relaxations of PCA stimulated by 10 μM histamine. The two columns at the left obtained from parallel experiments which show the height of the contractions induced by high K^+ and by histamine are included for the purpose of comparison. The error bars represent means values ± SEM and n = 6 for each column

The representative mechanogram shown in Figure 6 shows the reversal of glutamate-induced relaxations in contractions after a pre-treatment with 3,4-diaminopyrimidine. By itself 3,4-diaminopyridine elicited a strong phasic and unsustained contraction.

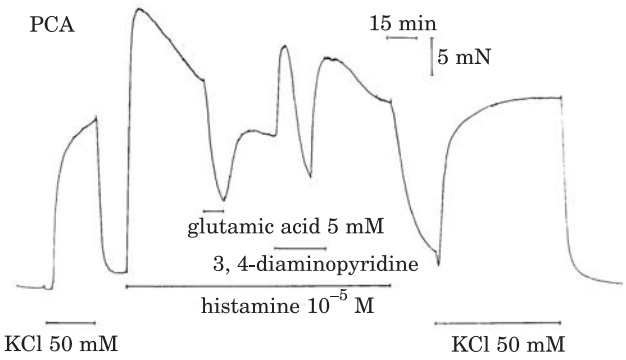

Fig. 6. Relaxing effect of glutamate (5 mM) on PCA preparations stimulated by histamine (10 μM) and its reversal to contraction after a pre-treatment with 3, 4-diaminopyrimidine (5 mM).
The preparations were contracted at the beginning and at the end of the experiment in order to test their reactivity.

5. Effects of glutamate on PCA preparations contracted by histamine in presence and in absence of glycine

It has been reported that glycine, which represents an essential coagonist, is indispensable for the physiological activation of NMDA receptors (Danysz, Parson 1998). In agreement with this requirement, the ED_{50} values of CCR curves obtained for the relaxation induced by L-glutamate in histamine-stimulated PCA preparations were shifted leftward from 0.8 mM to 0.25 μM in presence of 1 mM glycine (Figure 7).

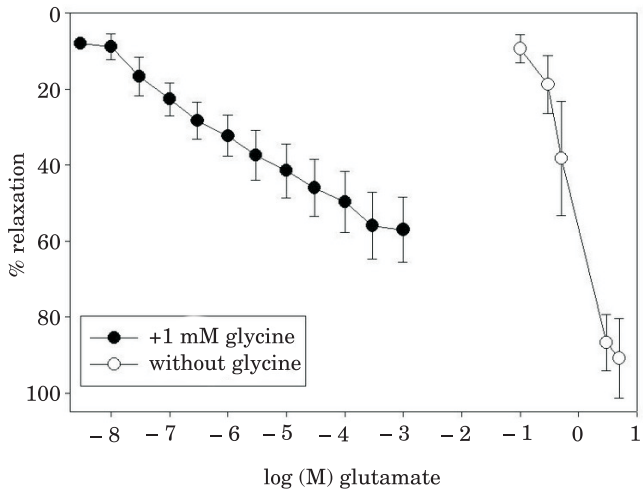

Fig. 7. Effects of glutamate on PCA preparations contracted by histamine in presence and in absence of glycine. The error bars represent means values ± SEM; n = 12.
See text for further details

6. Effects of glutamate on F^--induced PCA contractions and complete loss of contractility elicited by administration of L-glutamate on PCA preparations overactivated by treatment with F^- -ions

Depending of the level at which it is applied in the signal transduction chain, glutamate might exert quite different effects. CCR curves established for contractions induced by depolarization with K_o (10-100 mM) were slightly and significantly shifted to the right in presence of 5 mM glutamate (Figure 8, the left diagram), suggesting that glutamate-induced relaxations partly rely on a reduction of Ca entry. By contrast, the effects elicited by glutamate on F^--induced contractions were more complex (Figure 8, the right diagram). It has been shown in a previous study (NGUYEN-DUONG 1994) that fluoride ions, which form spontaneously $[AlF_4]^-$ (fluoroaluminate) are able to stimulate the signal transduction chain downstream of the receptor site by

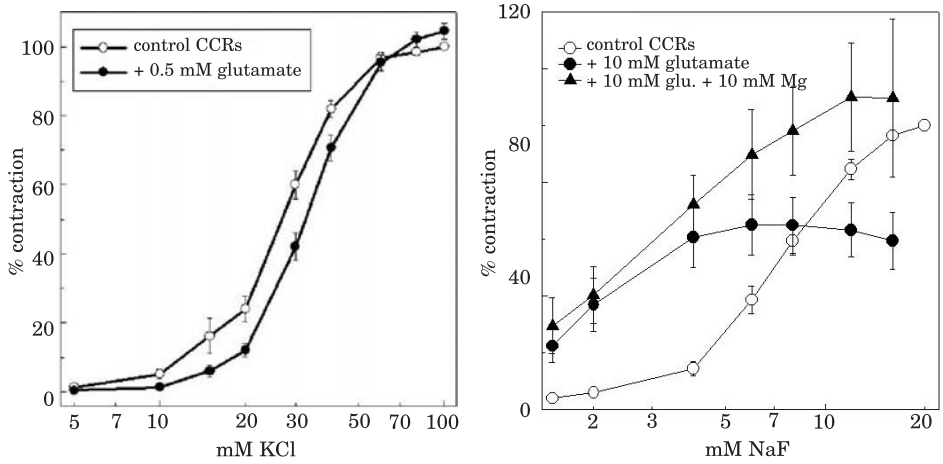

Fig. 8. Comparison of glutamate effects on contractions induced by cumulative increasing concentrations of external KCl (right) and of NaF.
Left diagram: 100% = 24.56 ±1.73 mN mm^{-2}, n = 12. Right diagram: 100% = 14.24 ± 1.52 mN mm^{-2}; n = 12. The errors bars represent means values 24± S.E.M. See text for further details

acting at the level of inositide signalling a pathway as a phosphate analogue with G proteins. In keeping with the assumed mechanism, the CCR curves obtained for contractions induced by NaF (1.5-12 mM) were significantly shifted to the left (ED_{50} from 6.3 to 3.1 mM), indicating that the sensitivity of contractions triggered by direct activation of G-proteins was increased in the presence of glutamate. Parallel to the leftward shift of the CCR curves, a depression of the maximum effect (reflecting a reduced intrinsic efficacy) was also observed at higher F^- concentrations (Figure 8, the right diagram). This observation may be explained as follows: since NMDA ion channels are subject to voltage-dependent Mg^{2+} blockade, a moderate depolarization elic-

ited by F^- would displace the channel-bound Mg^{2+} ions, leading in turn to an opening of channels associated with glutamatergic receptors and most probably to increased K^+ efflux. The ensuing hyperpolarization would lead to a reduced Ca entry and to inhibition of the vascular tone. Consistent with the proposed mechanism, this depression could be reversed by increasing the external Mg^{2+} to 5 mM (Figure 8 the right diagram).

When L-glutamate (10 mM) was added to F^--stimulated PCA preparations, within 1 to 2 hours a complete and irreversible loss of contractility and reactivity toward depolarization by 50 mM K^+ or applied NaF was regularly observed (not shown); control experiments performed in absence of L-glutamate demonstrated that contractility and reactivity of the PCA preparations remained completely intact within the same lapse of time (not shown).

DISCUSSION

The results presented in this study suggest that substances known for their ability to activate glutamatergic receptors in the CNS have pronounced relaxing effects on preparations excised from PCA. Although only some sporadic information exists on pharmacological effects of amino acids on the vascular smooth muscle, to my knowledge there is as yet no published report on receptors stimulated by binding of NMDA, GABA or kainate in PCA. Hence it is not possible at the moment to interpret the present results on the basis of available biochemical, molecularbiological or electrophysiological data. The first conspicuous discrepancy encountered in the pharmacological behaviour of the PCA preparations is the relaxation in response to the application of agonists of glutamatergic receptors, which has been recorded throughout and without exception. This finding is in contrast with the fact that in the CNS amino acids are excitatory by nature and that stimulation of NMDA receptors regularly leads to Ca entry in the postsynaptic cells. A logical inference is that the glutamatergic receptors involved in PCA are associated with K^+ channels. This assumption is consistent with the reversal of glutamate-induced PCA-relaxations observed after addition of a specific K^+ blocker (Figures 5 and 6). Glutamate seems also to unspecifically interfere with Ca entry through voltage-gated channels; however, the effects were transient, whereas the corresponding effects recorded after stimulation of ligand-operated receptors were sustained and of a larger amplitude (Table 1 and Figure 1). An additional hint for the hypothetical occurrence in the vascular wall of glutamatergic receptors of unidentified subtype is the extreme sensibilization of the glutamate-induced effects by glycine, a co-agonist of NMDA-receptors in the CNS (Figure 7) – NGUYEN-DUONG 2001. It has been postulated that Zn^{2+}, a co-factor and structural component of many

enzymes, which also plays a role as an intercellular signalling messenger, may be partly responsible for the neuronal death associated with transient global ischemia, with sustained seizures and with some neurological diseases (BENVENISTE et al. 1984). The different effects of Zn^{2+} described in this study seem to be consistent with its function as an endogenous modulator of ligand- and voltage-gated ion channels (Figures 3 and 4). A very strong argument for a modulator role of Zn^{2+} ions in ligand-gated receptors is their ability to inhibit histamine-stimulated PCA preparations, whereas the corresponding effects induced by acetylcholine were unaffected (Figure 4). Inside channel proteins Zn^{2+} ions coordinate to histidine, cysteine, aspartate and glutamate residues; since histamine is a derivative of histidine, the finding described in Figure 4 might not be wholly casual.

Because of the vital role played by zinc ions in the metabolism of proteins, carbohydrates, lipids, as well as in gene transcription, immune response, and many other fundamental biological processes, this essential trace element is strongly controlled by various homeostatic mechanisms, regulating their absorption, their cellular uptake and their distribution among intracellular compartments. On account of the extreme efficacy of the underlying homeostatic mechanisms, the nontoxicity of even excessive zinc ingestion has been generally taken for granted (VALLEE, FALCHUK 1993). However, recent research demonstrating that dysregulation of zinc and copper homeostasis in the brain might play an extremely critical role in Alzheimer disease (MELONI et al. 2007) emphasize the necessity of re-evaluating this far too simplistic assumption made on the innocuousness of excessive zinc (VALLEE, FALCHUK 1993) and support precautions to be taken with regard to zinc as an environmental toxicant and as a cerebral neurotoxin. In terms of integrated systems with regulatory functions, however, the pharmacological and toxicological significance in the organism of copper and zinc ions is only understood when these ions are considered by pairs, as reciprocal antagonists, in the same way as it is done with calcium and magnesium ions.

The relaxing effect of glutamate described in the present study may be linked to the well-known so-called Chinese restaurant syndrome, which manifests itself as headaches and flushing (OLNEY 1990). The ED_{50}, i.e. the dose producing a half-maximum relaxation in PCA preparations amounted to about 0.9 mM. This value is of the same order of magnitude that reported in plasma of humans after an experimental ingestion of monosodium glutamate, in which the concentration peaked until a value of about 0.5 mM (GRAHAM et al. 2000).

A „cytotoxic" effect of glutamic acid has been also demonstrated in an isolated rat lung (SAID 1999), which seems to correspond to that observed in neurones and glial cells and can be related to the neurotoxic effect described in the CNS. The observation made in the present study that a high concentration of L-glutamate (10 mM) when added to F^--stimulated PCA preparations led to a complete and irreversible loss of contractility and reactivity

might be explained as follows: stimulation with fluoride ions takes place downstream of the receptor site and represents a rather unspecific intervention that targets several classes of G proteins, impinging upon several effector systems; together with a fluoride-induced intracellular Ca overload, this would in turn inevitably lead to a rather indiscriminate stimulation of proteases, protein kinases and phospholipases. An activation of phospholipase A_2 and of cyclooxygenase would for example generate free-radical species overwhelming the endogenous scavenging mechanisms and producing lipid peroxidation and membrane damage.

In conclusion, the data presented in this study, which give a preliminary account of the sensitivity of peripheral blood vessels to glutamate, to zinc ions and to related substances, need undoubtedly further electrophysiological investigations before therapeutic implications could be deduced. Nevertheless, the total loss of contractility of hyperstimulated blood vessels after the administration of glutamate reported in the present study let one speculate on initial stages in the pathogenesis of atherosclerotic lesions. It is conceivable that similar mechanisms might underlie permeability changes of the cell membrane, leading to local oedema of the blood vessels, favouring in turn the accumulation of lipids, the activation of macrophages and provoking eventually an irreversible production of foam cells.

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