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Simultaneous determination of cholecystokinin, dopamine, glutamate and aspartate in cortex and striatum of the rat using in vivo microdialysis

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Summary. Extracellular levels of cholecystokinin (CCK), dopamine (DA), glutamate (Glu) and aspartate (Asp) were simultaneously monitored in the fronto-parietal cortex and the striatum of halothane-anaesthetized rats using in vivo microdialysis. Under basal conditions, cortical and striatal CCK levels were 3.11 ± 0.39 pM and 2.76 ± 0.15 pM, respectively. Local KCl (10^{-1} M) and bicuculline (10^{-4} M) co-application in cortex or striatum increased the CCK levels 18-fold and 26-fold, respectively. The DA level in striatum was 3.78 ± 0.28 nM and the local perfusion with KCl + bicuculline led to a 45-fold increase. The cortical and striatal outputs of Glu were of the order of 2×10^{-6} M and Asp levels were around 6×10^{-7} M. Local stimulation with KCl (10^{-1} M) and bicuculline (10^{-4} M) caused a small increase (2 fold) in cortical and striatal levels of Glu and Asp. The addition of KCl (10^{-1} M) and bicuculline (10^{-4} M) to the cortical perfusion medium did not modify CCK, DA or Glu concentrations in striatum. These results demonstrate that CCK, DA, Glu and Asp may be simultaneously monitored in vivo and support the idea that their extracellular levels recovered in the microdialysis perfusates could be derived from neuronal pools.

Keywords: Amino acids – Glutamate – Aspartate – Dopamine – Cholecystokinin – Microdialysis – Basal Ganglia

Introduction

Excitatory amino acids (EAA), in particular L-glutamate (Glu) and L-aspartate (Asp) are major neurotransmitters in the mammalian cerebral neocortex (for a review, see Tsumoto, 1990). In addition, it is generally accepted that a direct glutamatergic projection from the cerebral cortex provides one of the major afferent inputs to the striatum (Graybiel and Ragsdale, 1979). In the striatum, interactions between the dopaminergic nigrostriatal pathway and the gluta-

matergic corticostriatal projections have been suggested. It has been shown that the release of dopamine (DA) is modulated by the glutamatergic cortical input involving mono- and poly-synaptic loops (Giorguieff et al., 1977; Nieoullon et al., 1978; Roberts and Sharif, 1978; Jhamansdas and Marien, 1987; Carlsson and Carlsson, 1990; Herrera-Marschitz et al., 1990) and that the release of Glu can also be regulated by dopaminergic nigrostriatal terminals (Rowlands and Roberts, 1980; Mitchell and Doggett, 1980; Godukhin et al., 1984; Crowder and Bradford, 1987; Maura et al., 1988; Reid et al., 1990).

Mesencephalic DA systems project to striatum, limbic structures and the neocortex. Indeed, evidence for a widespread dopaminergic innervation throughout the neocortex including the frontoparietal (sensoriomotor) cortex has been reported by using histochemical (Berger et al., 1985; Descarries et al., 1987; Yoshida et al., 1988; Papadopoulos et al., 1989) and microdialysis (Herrera-Marschitz et al., 1989; Maysinger et al., 1990; Reid et al., 1991) techniques. In all of these studies the DA innervation fields and detectable levels of DA appear to be restricted to the deep layers of the cortex.

Cholecystokinin (CCK) is a peptide neurotransmitter candidate that has been found in many areas of the rat brain including cortex and striatum (see Vanderhaeghen and Crawley, 1985). CCK coexists with DA neurons in the ventral tegmental area and the substantia nigra (Hökfelt et al., 1980; 1988) and is involved in the regulation of DA transmission (see Wang et al., 1984; Crawley, 1991). However, non-dopaminergic CCK terminals and probably intrinsic CCK inmunoreactive neurons have been also described in caudate nucleus (Meyer et al., 1982; Hökfelt et al., 1988). Recently, cortical neurons expressing CCK mRNA have been mapped showing that pyramidal neurons expressing the CCK mRNA are concentrated in layers II/III and V/VI (Burgunder and Young, 1990; Lindefors et al., 1990) and form long cortico-cortical and cortico-striatal projections (Burgunder and Young, 1990).

In order to further study the interactions between all these neurotransmitters, the simultaneous measurement of their release in vivo would be of great value. In the present study, we have therefore utilized in vivo microdialysis to detect and monitor simultaneously extracellular levels of CCK, DA, Glu and Asp in cortex and neostriatum of rats under basal and KCl stimulated conditions.

Material and methods

Surgical procedures and sampling collection

Sprague-Dawley male rats weighing 472 ± 8 g were anaesthetized with a mixture of air and halothane and placed in a David Kopf stereotaxic frame. After exposing the skull, two holes were drilled for the placement of the microdialysis probes into the lateral portion of the corpus of the left striatum (coordinates according to König and Klippel (1963): B 0, L +3.6, V −7.2, vertically implanted) and into the left frontoparietal cortex (coordinates: B +1.0, L +2, V −5.8, inserted with a 40° angle from vertical in the coronal plane). Halothane anaesthesia (1% in 1.5 l/min air flow) was maintained throughout the microdialysis experiment by free breathing into a mask. Body temperature was kept at 37°C using a temperature control system (CMA 150, Carnegie Medicin AB, Stockholm, Sweden).

The microdialysis probes (CMA 12, Carnegie Medicin AB) were perfused with a Krebs Ringer solution (NaCl 138 mM, $NaHCO_3$ 11mM, KCl 5 mM, $CaCl_2$ 1mM, $MgCl_2$ 1mM,

NaH_2PO_4 1mM, pH 7.4) including 2 g/l glucose, 2 g/l bovine serum albumin (BSA) and 0.3 g/l Bacitracin. The flow was maintained at a constant rate of 2 μl/min with a microdialysis perfusion pump (CMA 100, Carnegie Medicin AB), and 80 μl perfusate samples were collected every 40 min in 300 μl glass tubes. After 200 min collection period to determine basal levels, KCl (10^{-1} M) with bicuculline (10^{-4} M) were included in the Krebs Ringer perfusion medium and applied for 80 min in the cortex. In the striatum, the KCl (10^{-1} M) with bicuculline (10^{-4} M) was applied at the 360–440 min period after the implantation of the microdialysis probes. The GABA antagonist bicuculline was co-administered with KCl in the perfusion medium in order to inhibit the intrinsic GABAergic neurons and therefore to increase the activity of neocortical pyramidal cells projecting to the neostriatum. Changes in the perfusion medium were automatically performed with a syringe selector coupled to a microfraction collector (CMA 111, CMA 140, Carnegie Medicin AB). The probes used in this study showed a approximately 20% in vitro recovery for Glu, Asp and DA and a 10% for CCK (at room temperature).

Sample analysis

The determination of CCK concentrations was carried out as previously reported for the analysis of other neuropeptides (Brodin et al., 1983; Lindefors et al., 1987). Briefly, 100 μl of Krebs Ringer perfusion solution (containing 40 μl of perfusate samples) were incubated in Eppendorf polyethylene tubes for 24 h at 4°C with the specific C-terminal directed gastrin/CCK antiserum 2609 (Rehfeld, 1978). After subsequent addition of [^{125}I]iodogastrin (Human Gastrin I; 1000 cpm/tube) (Milab, Malmö, Sweden) all the samples were incubated for a further 72 h at 4°C. Diluted antiserum and radioligand were added in barbital buffer to yield a final concentration of 10^{-1} M. The incubation medium also contained 2g/l BSA and 0.3 g/l Bacitracin. Simultaneously peptide standards of CCK-8 (3×10^{-13} M -1.8×10^{-10} M, ten concentrations) (Peninsula, Belmont, CA, USA), samples without unlabeled peptide (maximal tracer binding) and samples without antibody (non-specific binding) were also incubated. After the incubation procedure, antibody-bound and free tracer were separated by antirabbit Ig G coupled to sepharose (Pharmacia Decanting Suspension, Pharmacia, Uppsala, Sweden) and centrifugation at 2000 $\times$ g during 10 min. The EC_{50} (concentration of CCK-8 inhibiting by 50% the tracer binding to the antiserum in the standard curve) was 8.14 $\pm$ 1.56 pM and the limit of detection for CCK was below 1 pM. The antiserum dilution was adjusted to obtain approximately 30% specific binding of the [^{125}I]iodogastrin in the absence of unlabeled peptide. The non-specific binding of tracer to the precipitated pellet in the absence of the antiserum was less than 2%.

Glu and Asp concentrations in the perfusates were measured by high performance liquid chromatography (HPLC) using precolumn derivatization with o-phthaldialdehyde/mercaptoethanol (OPA) reagent and fluorometric detection. Briefly, 10 μl of the OPA reagent (0.4 M borate, 0.04 M o-phthaldialdehyde, 0.4 M 2-mercaptoethanol, pH 10.4) was added to 10 μl of perfusate and after 60 s reaction time, 15 μl of the derivatizated aliquot were injected into a column (100 $\times$ 3.2 mm) prepacked with Biophase ODS 5-μm particles (BAS). Derivatization was made in an autosampler (CMA 200, Carnegie Medicin AB) at 4°C. The eluent was a mixture of 0.1 M sodium acetate, 9% methanol adjusted to pH 6.95 and 1.5% tetrahydrofurane (THF). The eluent was pumped by a pump equipped with a two way valve (SP 8800-020, Spectra-Physics, San Jose, CA, USA) at a flow of 1 ml/min. A multilinear gradient was used (100% eluent changing to 100% methanol over 6 min and returning to 100% eluent 2 min later). The fluorometric detector was a Hitachi F1000 (Hitachi Ltd., Tokyo, Japan) with excitation wavelength set at 370 nm and an emmission cut-off filter set at 450 nm. The detection limit of the compounds measured was 10 nM.

A reverse-phase ion pair HPLC combined with electrochemical detection (BAS, West Lafayette Inc., USA) was used for the measurement of DA as previously reported (Reid et al., 1988). The column (250 $\times$ 4.6 mm) was prepacked with Biophase ODS 5-μm particle size material (BAS). The mobile phase contained 0.10 M NaH_2PO_4, 0.08 M EDTA, 1.5 mM 1-octanosulphonic acid in a 15% methanol solution, pH 3.8. Electrochemical detection was

done using a glassy carbon electrode (BAS LC-4A) set at 0.65 V. The detection limit for DA was 0.1 nM.

Histological procedure

After dialysis experiments, the rats were killed with a halothane overdose and their brains rapidly dissected and stored in 10% formalin for histological analysis of the location of the microdialysis probes and verification of the tissue damage. Sections (30 μm) were cut on a freezing microtome. In all animals the dialysis probes were appropiately located in the fronto-parietal cortex and in the lateral portion of the corpus striatum.

Statistics

Results are expressed as the mean $\pm$ SEM of the concentration values or percentages from perfusate samples obtained under basal conditions. Student's t-test was used and a level of $P < 0.05$ (two tails test) was considered as critical for statistical significance.

Results

The highest levels of CCK, DA, Glu and Asp before the stimulation periods were seen during the 0–40 min perfusion period, i.e., immediately after the implantation of the probe. In the cortex, extracellular levels of Glu and Asp decreased slowly along the perfusion course, while DA and CCK levels disminished rapidly in the striatum, where the levels remained steady during the second collection period (40–80 min) (Fig. 1 and 2). In some cases, the basal levels of CCK were below the limit of detection.

At the 160–200 min period (basal conditions) the mean extracellular levels of CCK were 3.11 $\pm$ 0.39 pM in cortex (detectable levels in 5 out of 7 experiments) and 2.76 $\pm$ 0.15 pM in striatum (detectable levels in 6 out of 7 experiments). Under the local stimulation with the depolarising agent KCl (10^{-1} M) and the GABA antagonist bicuculline (10^{-4} M) diluted in the Krebs Ringer used as perfusion medium, CCK levels were increased to 57.6 $\pm$ 20.7 pM (+1800%; n = 7) and to 73.4 $\pm$ 19.3 pM (+2600%; n = 7) in cortex and striatum, respectively. The levels of CCK remained elevated during the second period of stimulation. In cortex the CCK level returned slowly to the control values when the KCl was removed from the perfusion medium (Fig. 1 and 2). In striatum, the microdialysis experiment was terminated after the second period of stimulation with KCl (Fig. 2).

In the striatum, the basal DA levels (160–200 min period) were 3.78 $\pm$ 0.28 nM and were increased by 45 $\pm$ 15 fold following KCl + bicuculline stimulation (Fig. 2). No reliable measurement of cortical DA could be done under basal conditions, but following KCl (10^{-1} M) and bicuculline (10^{-4} M) stimulation, cortical DA levels were 4.85 $\pm$ 1.07 nM ($n = 6$).

Cortical basal values of Glu, refered as those obtained at the 160–200 min period, were 1.96 $\pm$ 0.41 μM ($n = 7$) and did not differ from those obtained in striatum (2.38 $\pm$ 0.60 μM; $n = 7$) during the same period. Simultaneous determination of Asp displayed extracellular values of 0.59 $\pm$ 0.08 μM ($n = 7$) in cortex and did not differ from those obtained in the striatum (0.56 $\pm$ 0.25 μM). Stimulation in the cortex with KCl (10^{-1} M) containing bicuculline (10^{-4} M) increased

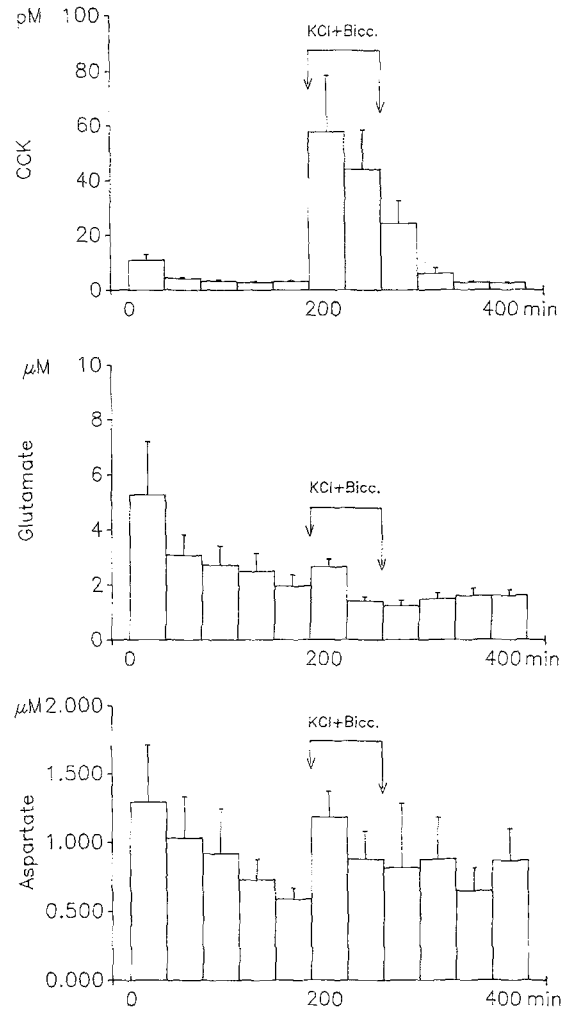

Fig. 1. Graphical representation of the time course for cholecystokinin, glutamate and aspartate levels in samples collected simultaneously from the left fronto-parietal cortex of 3–7 normal rats. Bars represent the mean values ± standard error (vertical lines) of extracellular levels determined in perfusate fractions of 40 min perfusion fractions. The abscissa indicate the time (min) after the implantation of the microdialysis probe. During the 200–280 min period (2 fractions) KCl (10^{-1} M) + bicuculline (10^{-4} M) were included in the perfusion medium

both Glu (165 ± 10%) and Asp (232 ± 15%; $p < 0.05$) levels in the ipsilateral cortex (although the change on Glu did not reach a statistical significant level). In the striatum, the inclusion of KCl (10^{-1} M) and bicuculline (10^{-4} M) into the perfusion medium (360–440 min period) increased Glu and Asp levels by 236 ± 28% ($p = 0.069$) and 237 ± 83% ($p = 0.034$) respectively (Fig. 1 and 2).

The microinfusion of KCl (10^{-1} M) and bicuculline (10^{-4} M) into the cortex through the microdialysis probe did not alter the extracellular levels of Glu, DA or CCK in striatum (c.f. Fig. 1 vs Fig. 2). Striatal Asp showed a small increase (153 ± 18%) in the values during the second period of cortical stimulation by KCl and bicuculline (240–280 min after implantation) but this result was not statistically significant (Fig. 2).

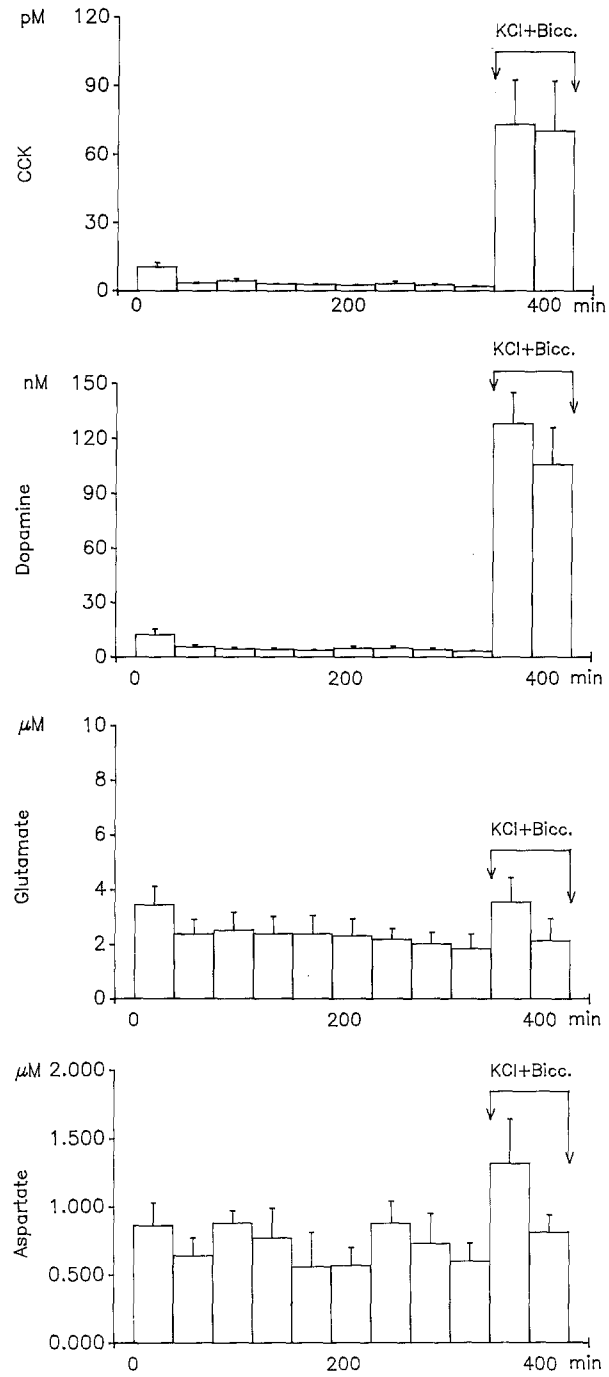

Fig. 2. Graphical representation of the time course for cholecystokinin, dopamine, glutamate and aspartate levels in samples collected simultaneously from the left neostriatum of 3–7 normal rats. Bars represent the mean values ± standard error (vertical lines) of extracellular levels determined in perfusate fractions of 40 min perfusion fractions. The abscissa indicate the time (min) after the implantation of the microdialysis probe. During the 360–440 min period (2 fractions) KCl (10^{-1} M) + bicuculline (10^{-4} M) were included in the perfusion medium

Discussion

The present study demonstrates that extracellular cortical and striatal CCK levels may be simultaneously monitored in vivo, using microdialysis in halothane-anaesthetized rats, under basal and K^+-depolarizating conditions. Furthermore, DA and the EAAs Glu and Asp could be also detected. In order to increase the recovery of CCK, a perfusion medium including BSA and Bacitracin was used, which, however, produced some chromatographic interferences for the detection of DA.

The neuropeptide CCK was detected in cortex and striatum showing constant and reproducible basal levels. This data is partly in contrast to the finding of De Mesquita et al. (1990), who have reported limitations for the detection under basal conditions of extracellular CCK in the nucleus accumbens by microdialysis and a radioimmunoassay for CCK-8 sulfate. Their low recovery reported (0.1–2.4%) could contribute to these constrasting findings. The release of cortical and striatal CCK appears to involve depolarization-induced exocytosis, since local KCl stimulation dramatically increased CCK output in the dialysate. Our findings are compatible with the observations of Takita et al. (1989) who using microdialysis and an enzyme immunoassay, described cortical (prefrontal region) CCK basal levels in dialysates around 35 pM and a Ca^{2+}-dependent, KCl-induced CCK release in this cerebral area. The present microdialysis study confirms the presence of high levels of CCK-like immunoreactivity in the caudate nucleus (Hökfelt et al., 1988) and in the frontoparietal cortex (see Vanderhaeghen and Crawley, 1985) of rat brain. In the present study, cortical stimulation by KCl and bicuculline did not induce an increase of CCK levels in the ipsilateral striatum. Therefore, the issue whether or not CCK is released from a corticostriatal CCK pathway (Burgunder and Young, 1990) remains to be studied.

Extracellular levels of Glu and Asp were found in striatum in the same micromolar range to those previously reported using microdialysis technique (Young and Bradford, 1986; Butcher and Hamberger, 1987; Westerink et al., 1989; Palmer et al., 1989). Although no previous data on cortical Glu and Asp levels by microdialysis technique are available, similar concentrations of Glu and Asp in cortex and striatum have been established by other methods (Erecinska and Silver, 1990; Tsumoto, 1990). In response to local administration of KCl and bicuculline, cortical or striatal extracellular concentrations of Glu and Asp exhibited a small increase, estimated to be around 2 fold the basal levels, which makes it difficult to assess the origin of these amino acids. However, a selective loss of basal and veratrine-releasable Glu and Asp has been found in striatum after decortication suggesting that a high proportion of the extracellular pools of Glu and Asp are derived from a neuronal pool (Butcher and Hamberger, 1987). Similar effects were described for the depolarizing toxin tityustoxin on striatal release of Glu and Asp (Young and Bradford, 1986). Moreover, we have recently found that a larger KCl-stimulated release of Glu and Asp is seen following the inclusion in the perfusion medium of the EAA selective uptake blocker dihydrokainate (10^{-3} M) without changes in basal values (Herrera-Marschitz et al., 1991; Meana et al., 1991). Therefore, the detected extracellular

concentration of the Glu and Asp is likely to be related to neuronal activity and high-affinity uptake mechanisms for EAA existing in neurons and glial cells.

The levels of DA detected in the fronto-parietal cortex under KCl stimulation were approximately similar to those detected in the striatum under basal conditions. Previous studies in this laboratory have shown that values of cortical DA were around 1/10 of those obtained in striatum and that KCl (10^{-1} M) increases DA concentrations in cortex 8 fold, while in the striatum they were increased 25 fold (Herrera-Marschitz et al., 1989; Maysinger et al., 1990; Reid et al., 1991). The reason for the difficulty in measuring cortical DA levels remains to be clarified, but the use of different perfusion media (Ringer buffer in the previous and modified Krebs-Ringer including BSA and Bacitracin in the present study) might contribute to this difficulty.

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