Cyclopropyl Glycine and Proline-Containing Preparation Noopept Evoke Two Types of Membrane Potential Responses in Synaptoneurosomes

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Proline, cyclo(Pro-Gly), and acyl-prolyl-containing dipeptide GVS-111 decreased synaptoneurosome membrane potential in a Ca^{2+}-free medium. The efficiency of these preparations decreased in the following order: GVS>cyclo(Pro-Gly)>proline. Depolarization responses induced by endogenous nootropic agent cyclo(Pro-Gly) was dose-dependent and saturable; the threshold concentration of cyclo(Pro-Gly) was 10^{-9} M. In a Ca^{2+}-containing medium GVS and cyclo(Pro-Gly) induced both hyperpolarizing and depolarizing membrane responses of synaptoneurosomes. Possible mechanisms underlying changes in the membrane potential of synaptoneurosomes induced by nootropic agents are discussed. It was interesting whether modulation of electrogenesis can improve memory and potentiate the neuroprotective effect of the test nootropic agents.

Key Words: *proline-containing nootropic agents; synaptoneurosomes; membrane potential; memory; neuroprotection*

A nootropic dipeptide cyclo(Pro-Gly) (CPG) was recently found in the brain of animals. The structure and pharmacological properties of CPG are similar to those of synthetic nootropic agent piracetam [2]. These data suggest that the pharmacological effects of racetams are determined by their interaction with receptors for endogenous nootropic agents. The preparation GVS-111 (GVS, Noopept, ethyl ester of N-phenylacetyl-L-prolylglycine) synthesized from piracetam more effectively improved learning and produced a more pronounced neuroprotective effect than CPG and piracetam [4]. GVS prevented neuronal death in culture induced by various pathogens. Moreover, GVS alleviated cytotoxic consequences of focal ischemia in rats [1,4].

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In mammals ion channels are a possible target for nootropic peptides in neurons. The physiological effects of these peptides (modulation of memory and neuroprotection) can result from permanent changes in the membrane potential. Synaptoneurosomes (SNS) are a convenient model for studying changes in the membrane potential caused by changes in membrane permeability, because they are characterized by higher surface/volume ratio compared to neurons. Here we evaluated the effects of memory-modulating compounds proline, CPG, and GVS on membrane potential of SNS.

MATERIALS AND METHODS

Isolation of SNS and membrane potential recordings with DiS-C_2-(5) (Molecular Probes Inc.) were performed as described elsewhere [7,9]. Experiments were performed on male outbred rats weighing 90-100 g. The animals were decapitated under ether anesthesia. The brain was placed in an ice-cold medium, the pia

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mater was removed, the cortex was dissected and cut with scissors. Tissue fragments were placed in a cold glass-Teflon homogenizer with isolation medium and homogenized (6-8 slow frictions). Tissue concentration in the homogenate was 0.1 g/ml. The isolation medium contained 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 20 mM TES buffer (pH 7.4, NaOH). All reagents were from Merck. The homogenate was filtered through a double layer of synthetic fabrics (pore size 133 μ) and Millipore filters SCWP 02500 (pore size 8 μM) or LCWP 04700 (pore size 10 μ). The filtrate was centrifuged in a K-24 centrifuge at 2500*g* for 30 min. The supernatant was removed, and the pellet was resuspended. Equal volumes of suspension were placed in centrifuge tubes and centrifuged. The pellets were coated with freshly prepared isolation medium and stored in a refrigerator on ice.

For membrane potential recordings the pellet was resuspended in the isolation medium. The suspension (50 μl) was transferred to a thermostated cuvette with 2 ml incubation medium (isolation medium+2 μM DiS-C_2-(5)+10 mM glucose, 37°C). The concentration of Ca^{2+} in the medium was 0 (without Ca^{2+}), 1.2 (normal), or 3.0 mmol depending on the objective of measurements. Probe absorption was estimated on a Hitachi-320 double-beam spectrophotometer or Hitachi-557 two-wavelength spectrophotometer at 590 and 646 nm. During measurements on a Hitachi-557 spectrophotometer the potential-dependent difference in absorption at 590 and 646 nm was recorded, which allowed evaluation of the kinetic characteristics of the response. Gramicidin (2 μM) was added by the end of each series of measurements to decrease the membrane potential. The difference between values determined before and after addition of gramicidin (gramicidin-induced response) was taken as the resting potential. The effects of compounds were expressed in percents of the gramicidin-induced response. Experiments with membrane depolarizes KCl, Ba^{2+}, and 4-aminopyridine (4-AP) showed that potential-sensitive absorption of DiS-C_2-(5) (difference between absorption at 590 and 646 nm) decreases during depolarization of SNS [7,9].

Protein concentration in a cuvette was measured by the method of Lowry. The significance of differences was estimated by nonparametric Mann—Whitney test.

RESULTS

Proline, CPG, and GVS shifted the membrane potential of SNS. The directionality, amplitude, and dynamics of changes depended on the conditions of SNS storage, SNS protein content in a cuvette, ionic composition of the incubation medium, and concentration of preparations. In a Ca^{2+}-free medium the addition of proline-containing preparations to a cuvette produced depolarizing changes in the membrane potential with superimposed rapid oscillations (Table 1, Fig. 1, *a-c*).

At low concentration of preparations (10^{-9}-10^{-6} M) the response developed and decayed within several minutes or manifested as a flash of rapid oscillations of membrane potential (Fig. 1, *a-c*). After addition of CPG and GVS in higher concentrations the response appeared as a shift in the membrane potential toward a certain level (Fig. 1, *d*). In experiments with 3 compounds GVS was most potent, while proline was least effective in producing a shift in the membrane potential (Fig. 1, Table 1). Proline often caused rapid oscillations of the membrane potential (Fig. 1, *d*).

The interaction between CPG and GVS was competitive, rather than additive. CPG added after treatment with GVS and glucose abolished the shift in the membrane potential produced by GVS. We recorded only the first immediate shift and acceleration of rapid oscillations (Fig. 1, *d*). Slow changes in probe absorption were related to physicochemical properties of the probe, but not to the shift in the membrane potential (Fig. 1, *d*). These changes developed also in the absence of SNS. Double-beam measurements with the probe present in 2 cuvettes showed that the membrane potential of SNS was stable througout the experiment.

The effects of CPG and GVS were dose-dependent and saturable (Fig. 2, Table 1). Unresponsiveness to a further increase in CPG concentration in the sample did not abolish the effects of other depolarizing agents (*e.g.*, 4-AP, Fig. 1, *d*). Ba^{2+} (2 mM), 4-AP and, particularly, KCl (20 mM) induced greater depolarization of SNS membranes than proline-containing compounds.

The response of SNS to CPG and GVS in a Ca^{2+}-containing medium differed from that in a Ca^{2+}-free medium by the presence of a hyperpolarizing component (Fig. 3). GVS added to the cuvette after KCl caused depolarization, but not hyperpolarization of SNS (Fig. 3). Hyperpolarization in a Ca^{2+}-free medium was observed only when we used the first sample of SNS

TABLE 1. Depolarizing Effects of Test Compounds (% of Gramicidin-Induced Response, *M±m*)

Preparation	Concentration, M	Depolarizing response
CPG	3×10^{-5}	7.0±0.5 (13)
CPG	3×10^{-6}	3.2±0.8 (6)
GVS	3×10^{-6}	10.3±1.8 (3)
Glutamate	5×10^{-5}	6.7±1.1 (3)
KCl	23×10^{-3}	28.1±5.15 (3)

Note. Number of measurements is shown in brackets.

suspensions taken immediately after suspending of SNS stored as a dense pellet covered by isolation medium.

These data are the first proof of modulation of membrane potential in mammalian neurons by the endogenous nootropic agent CPG and preparation GVS. Depolarizing changes in the membrane potential of SNS caused by test nootropic agents can result from the blockade of K^+ channels, which was revealed during the action of GVS on snail neurons [6]. Membrane depolarization increases excitability of neurons, which is consistent with the ability of test compounds to stimulate learning [2,3]. The phenomenon of SNS membrane hyperpolarization can be selectively abolished by removal of Ca^{2+} from the incubation medium. Similar differences in the sensitivity of two types of Ca^{2+}-induced responses were observed during action of bradykinin on neurons. Hyperpolarization of neurons produced by bradykinin was abolished several minutes after treatment with the preparation in a Ca^{2+}-free medium. However, the depolarizing response in a Ca^{2+}-free medium remained unchanged [10]. GVS protect the organism from various pathogens [1]. For example, this agent prevents the development of ischemic stroke in animals [5]. Compounds opening K^+ channels and inducing membrane hyperpolarization in neurons possess these properties [8].

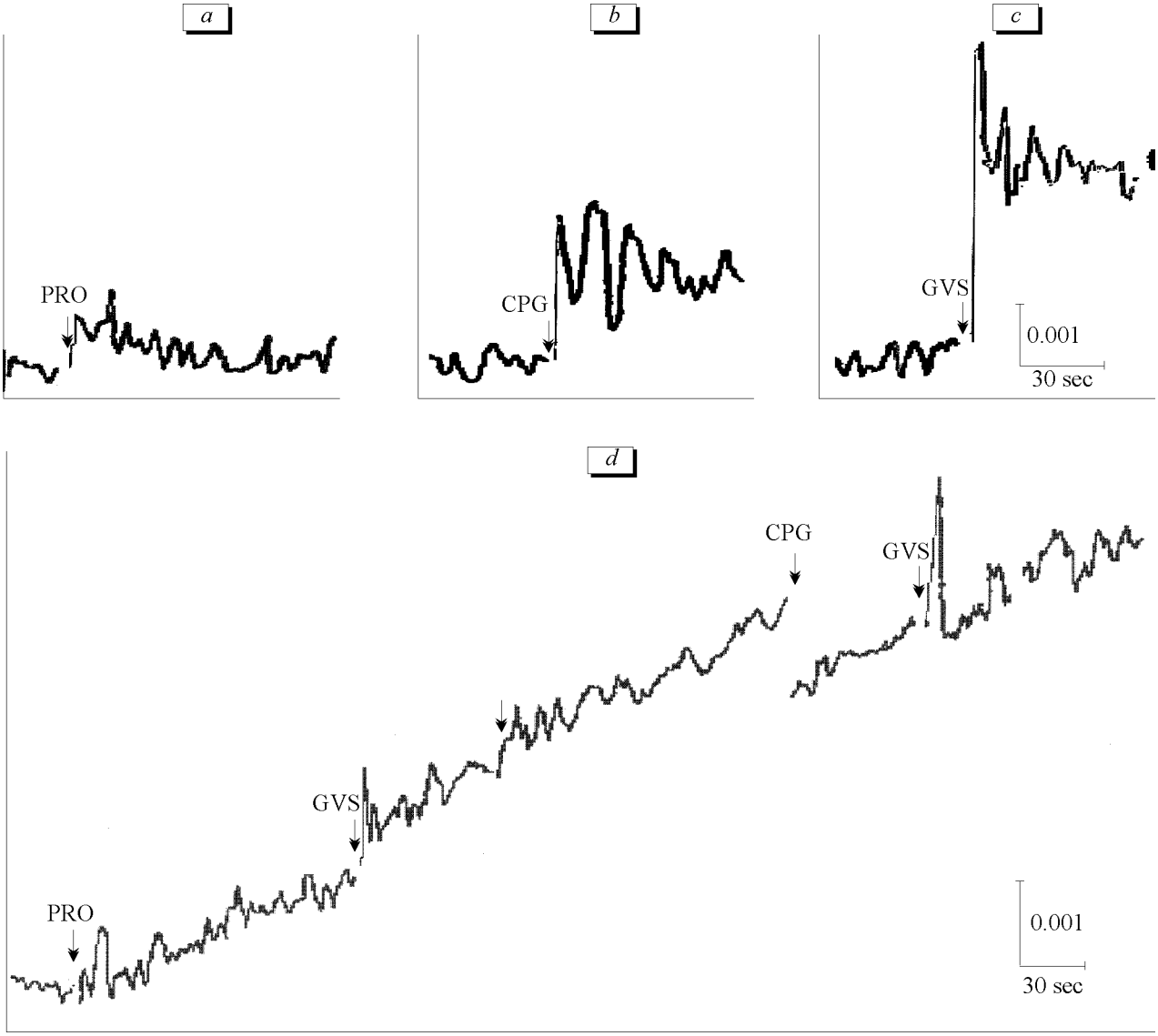

Fig. 1. Changes in optical density of probe DiS-C_2-(5) produced by proline (*a*), cyclo(Pro-Gly) (CPG, *b*), and ethyl ester of N-phenylacetyl-L-prolylglycine (GVS, *c*) in a concentration of 10^{-6} M; interaction of these preparations. Ca^{2+}-free incubation medium. Potential-dependent difference in absorption at 590 and 646 nm. Calibration of changes in optical density of probe DiS-C_2-(5) and scanning speed (sec): bottom right (*c*). Depolarization and hyperpolarization correspond to upward and downward deflections, respectively. Anterior front of responses (*a-f*): pen displacement after the start of recording. Final concentration of the probe in a cuvette: 2 μM. Synaptoneurosome (SNS) protein concentration in samples: 290 μg/ml.

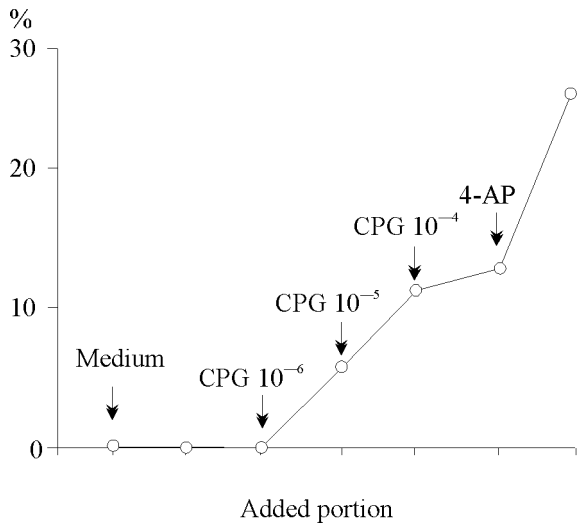

Fig. 2. Dose-response dependence for CPG. Ordinate: membrane depolarization in % of the gramicidin-induced response (resting potential of SNS). CPG concentration in a cuvette after treatment with the preparation is shown in Fig. 2. 4-Aminopyridine concentration: 1 mM.

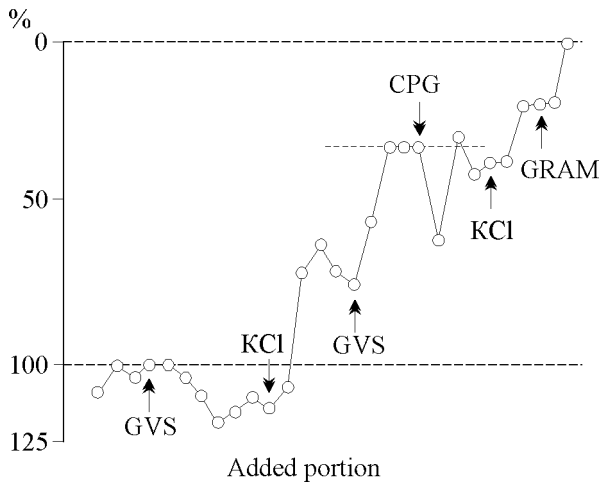

Fig. 3. Changes in the membrane potential produced by GVS and CPG (10^{-5}) in the medium with 3 mM Ca^{2+}. Final concentrations of KCl in a cuvette after the first and repeated treatment: 20 and 40 mM, respectively. Optical density: before addition of preparations, 100% membrane potential; 0, after addition of gramicidin (completely depolarized SNS).

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