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The unique molecular structure of the $\sigma_1$ receptor and its ligands belong to diverse structural classes, including benzomorphans ([+]-SKF 10.047 or (+)-N-allylnormetazocine, (+)-pentazocine), guanidines (1,3-di-o-tolyl-guanidine or DTG), butyrophenones (haloperidol), addictive drugs (cocaine, amphetamine), and certain steroids (e.g., progesterone, pregnenolone, or dehydroepiandrosterone) (for review, see Walker et al., 1990; Quirion et al., 1992). Two $\sigma$ binding sites have been identified pharmacologically. The $\sigma_1$ site binds (+)-benzomorphans, (+)-N-cyclopropylmethyl-N-methyl-1,4-diphenyl-1-ethyl-but-3-en-1-ylamine hydrochloride (JO-1784), $N,N$-dipropyl-2-[4-methoxy-3-(211phenylethoxy) phenyl]-ethylamine monohydrochloride (NE-100), DTG, and the antipsychotic haloperidol with high affinity, whereas the $\sigma_2$ site binds preferentially DTG and haloperidol (Walker et al., 1990; Junien et al., 1991; Quirion et al., 1992; Okuyama et al., 1993). It has been proposed that the $\sigma_1$ receptor is closely related to higher brain functions, including learning and memory, craving for drugs of abuse, psychotic and affective disorders, and neuroprotection by modulating neuronal excitability, especially via N-methyl-d-aspartate (NMDA)-related glutamatergic neurotransmission (Quirion et al., 1992; Su, 1993; Bowen, 1994).

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apparent lack of intrinsic activity (Hanner et al., 1996) has raised intriguing questions about its significance. Indeed, once it has bound its ligand, the $\sigma_1$ receptor, which at rest is anchored in the endoplasmic reticulum membrane, translocates to the plasma membrane and recruits calcium-dependent protein kinase C (PKC) isoforms (Novakova et al., 1998; Morin-Surun et al., 1999). Simultaneously with these molecular events, $\sigma_1$ receptor ligands either enhance [directly (Bowen, 1994) or indirectly via the bradykinin receptor (Hayashi et al., 2000)] or reduce [Ca$^{2+}$], via an inhibition of both NMDA receptor-associated (Fletcher et al., 1995; Whittemore et al., 1997) and voltage-sensitive calcium channels (Church and Fletcher, 1995)]. Whereas the latter inhibitory effect of $\sigma$ drugs on [Ca$^{2+}$], does not involve the $\sigma$ receptor (Fletcher et al., 1995; Whittemore et al., 1997), the studies documenting the potentiation by $\sigma_1$ drugs were carried out in immortalized cells but not in neurons after a $\sigma_1$ drug preincubation. The present study was therefore carried out in rat hippocampal pyramidal neurons to determine whether $\sigma_1$ drugs facilitate or not NMDA receptor-mediated influx of [Ca$^{2+}$], using successive brief concomitant applications of $\sigma_1$ drugs and glutamate by using microspectrofluorometry of the Ca$^{2+}$-sensitive indicator Fura-2 in primary cultures of embryonic neurons. To ascertain that the $\sigma_1$ receptor was involved, we have used the high-affinity and selective $\sigma_1$ receptor antagonist NE-100 (Okuyama et al., 1993).

Materials and Methods

Cell Culture. Procedures involving animals and their care were conducted in accordance with the guidelines of the Institut National de la Santé et de la Recherche Médicale Animal Use and Care Committee, which follows national and international laws and policies (EEC council directive 86/609). Pregnant Sprague-Dawley rats were purchased from Ifa-Credo (L’Arbresle, France).

Preparation of cultures of pyramidal neurons of the Ammon’s horn was adapted from the established methods used for rats (Banker and Cowan, 1977; Bartlett and Banker, 1984). Hippocampal formation from embryonic day 18 (E18) Sprague-Dawley embryo rats was isolated and mechanically dissociated by trituration in phosphate-buffered saline-glucose buffer (124 mM NaCl, 1.2 mM Na$_2$HPO$_4$, 1.25 mM KH$_2$PO$_4$, 4.7 mM KCl, and 11 mM glucose) and pipetting up and down with fire-polished Pasteur pipettes. After dissociation, the cells were centrifuged once (1000 rpm for 5 min, 4°C) to remove cellular debris, and cells were then plated onto sterile 25-mm-diameter poly-L-ornithine-coated glass coverslips (Marienfeld, Germany) at a density of $\sim 10^5$ cells/ml, seeded into six-well plates of 30-mm diameter with 1.5 ml of Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma Chimie, Saint Quentin Fallavier, France), 6 $\times$ 10$^{-5}$ M putrescine (Sigma Chimie), 25 $\mu$g/ml insulin (Sigma Chimie), 20 $\mu$m penicillin (Sigma Chimie), 0.02 mg/ml streptomycin (Sigma Chimie), 2.5 mM HEPES (Sigma Chimie), 3 $\times$ 10$^{-8}$ M sodium selenite (Sigma Chimie), 0.6% glucose (Sigma Chimie), 100 $\mu$g/ml streptovitron (Sigma Chimie) and maintained in a humidified incubator with 5% CO$_2$ at 37°C for 4 to 5 days before calcium imaging experiments.

[Ca$^{2+}$], Imaging by Microspectrofluorometry. At day 4 or 5 of culture, 12.5 $\mu$l of Fura-2/acetoxyethyl ester (AM) (final concentration of 2.5 $\mu$M; Grynkiewicz et al., 1985) was added for 30 to 45 min to the pyramidalically shaped neurons incubated with supplemented DMEM at 34°C under an atmosphere containing 5% CO$_2$. After the Fura-2/AM loading period, coverslips were washed once with modified Eagle’s medium (116 mM NaCl, 5.6 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM MgCl$_2$, 1.2 mM Na$_2$HPO$_4$, 5 mM NaHCO$_3$, 20 mM HEPES, and 11 mM glucose, pH 7.4). Dish coverslips were put into a thermostatically controlled holder on the stage of an Axiovert 35 microscope (Carl Zeiss, Jena, Germany) set up for epifluorescence microscopy, and immediately superfused with modified Eagle’s medium. The medium containing the hippocampal neurons was continuously renewed at a flow rate of 1.5 ml/min and the volume maintained constant at 150 $\mu$l, so that the time constant of the medium exchange was 6 s.

The solution was supplied by several inlet tubes, containing control and drug solutions, converging on the chamber. The temperature was kept at 34°C, because, in agreement with the findings of Kawashishi et al. (1989), intracellular Fura-2 fluorescence decreased rapidly at 37°C due to the dye being extruded from cells. [Ca$^{2+}$]$_i$ imaging was essentially as described by Clair et al. (2001). Briefly, the excitation light was supplied by a high-pressure xenon arc lamp (75 W), and the excitation wavelengths were selected by 340- and 380-nm filters wheel (10-nm bandwidth) mounted in a processor-controlled rotating filter (Sutter Instruments, Novato, CA) between the UV lamp and the microscope.

Fluorescence images were collected by a charge-coupled device camera (Princeton Scientific Instruments, Monmouth Junction, NJ), digitized, and integrated in real time by an image processor (MetaFluor, Princeton, NJ).

Procedures Assessing the Glutamate-Evoked [Ca$^{2+}$], Mobilization. A Ca$^{2+}$ increase was induced by successive brief (1 min) bath applications of $\sigma$ drugs, glutamate (Glu), or a combination of both via one inlet tube converging on the chamber. The temporal analysis of the Glu-induced [Ca$^{2+}$], variations was assessed from the digitized images collected every 3.0 s. Glutamate, which preferentially acts on the NMDA receptor in such a paradigm, was chosen because it allows a Ca$^{2+}$ entry that has been shown to subsequently trigger Ca$^{2+}$-dependent enzymes (e.g., PKC and calmodulin kinases) and [Ca$^{2+}$], release from the endoplasmic reticulum (Glau et al., 1990; Limbrick et al., 2001), but unlike NMDA, has lower acute toxicity for hippocampal neurons and does not trigger a long-lasting desensitization of NMDA receptors during our procedure of successive 1 min drug applications (Glau et al., 1990; Korkotian and Segal, 1996a,b; Limbrick et al., 2001; see Results). The first series of experiments was carried out to assess the concentration dependence of the glutamate-mediated [Ca$^{2+}$], influx via recruiting the NMDA receptor. The second series of experiments was carried out to assess the intrinsic activity of the $\sigma$ drugs on [Ca$^{2+}$],. The third series of experiments was carried out to assess the modulatory effects of the $\sigma$ drugs on the glutamate response, glutamate, and each of the $\sigma$ drugs being applied simultaneously on the chamber. To assess the involvement of protein kinase C in the $\sigma_1$ receptor-mediated effect and subsequent neuronal desensitization, the selective PKC inhibitor Go-6976 (200 nM), was incubated with cells for 30 min after the Fura-2 incubation and before the successive 1 min pulsates of $\sigma_1$ ligands and/or Glu.

Data Analysis. The Glu-induced [Ca$^{2+}$], increase in the presence of $\sigma_1$ ligands was compared with that in the absence of $\sigma_1$ drugs and expressed as the fluorescence ratiometric $\Delta$R/R$_0$ of fluorescence. Two types of analysis were carried out. The first one consisted in measuring the peak amplitude of the [Ca$^{2+}$], increase and was expressed as $\Delta$R/R$_0$. The second one corresponded to the integrated $\Delta$R/R$_0$, as a function of the total amount of the induced [Ca$^{2+}$], increase, with time and was expressed as $\Sigma$R/R$_0$.

The mean values (±S.E.) were obtained from independent coverslips obtained from 29 separate batches of cultures representing one experiment ($n$ given in parentheses). For each coverslip, between 5 and 12 neurons were recorded. Statistical significance between treated groups of hippocampal pyramidal neurons was assessed using the paired Student’s t test. Probability values smaller than 0.05 was considered as significant.

Chemicals. (+)-Pentazocine was a generous gift from Dr. W. D. Bowen (National Institute of Diabetes and Digestive and Kidney Diseases-National Institutes of Health, Bethesda, MD), (+)-N-alllylnormetazocine ((+)SKF-10,047) and JO-1784 were generous gifts.
from Dr. F. J. Roman (Institut de Recherche Pfizer-Joueival, Fresnes, France), NE-100 (N,N-dipropyl-[2-[4-methoxy-3-(211phenylethoxy)phenyl]-ethylamine monohydrochloride was a generous gift from Dr. S. Okuyama (Taisho Pharmaceutical Co., Tokyo, Japan), and glutamate (Sigma Chimie), MK-801 (dizocilpine; Sigma Chimie), and DTG (Sigma Chimie) were prepared extemporaneously and diluted in modified Eagle’s medium directly to the vicinity of the primary culture of hippocampal pyramidal neurons via the catheter. The protein kinase C inhibitor 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (Go6976) (Calbiochem, San Diego, CA) was prepared extemporaneously and incubated for 30 min with the primary culture of hippocampal pyramidal neurons before the recordings. Fura-2/AM was purchased from Molecular Probes (Leiden, The Netherlands).

Results

Glutamate-Mediated Increase in [Ca2+]i. The Glu-mediated [Ca2+]i signals were examined in cultured rat hippocampal pyramidal neurons at concentrations ranging from 0.5 to 500 μM (Fig. 1, A and B). To construct the concentration-response relationship of the Glu-induced [Ca2+]i, increase, successive 1-min pulses of Glu, using alternately increasing and decreasing concentrations of this dicarboxyl excitatory amino acid (to exclude any desensitization process; Cheng et al., 1999; Fink et al., 2000), were carried out. No fading of the glutamate response was noted whatever the glutamate perfusion procedure and Glu-induced increases in [Ca2+]i, were concentration-dependent and saturated at concentrations greater than 200 μM (EC50 = 111 μM considering ΔR/ΔR0 and EC50 = 115 μM considering ΔAR/ΔR0) from three independent experiments; Fig. 1B) in accordance with previous reports (Kudo and Ogura, 1986; Glaum et al., 1990; Cornell-Bell and Thomas, 1991; Limbrick et al., 2001). As shown in Fig. 1, A, C, and D, when 50 μM glutamate was used, the initial increase of [Ca2+]i was rapid (within less than 15 ± 3 s, from five independent experiments) followed by a plateau whose mean duration was 48 ± 12 s preceding a regular decrease to the baseline signal (mean duration of 117 ± 55 s), also consistent with previous reports (Kudo and Ogura, 1986; Glaum et al., 1990; Witt et al., 1994). When MK-801 (0.5 μM), a noncompetitive NMDA receptor antagonist, was bath-applied, it inhibited Glu-induced [Ca2+]i influx (Fig. 1C from three independent experiments). Removing Ca2+ from the perfusion buffer for 5 min inhibited the response to glutamate (Fig. 1D from five independent experiments) and indicates that the [Ca2+]i response resulted predominantly from extracellular Ca2+ influx.

For the subsequent experiments, the concentration of glutamate was 50 μM, which corresponds to ~40% of the maximal response to glutamate (Fig. 1B).

Effect of σ1 Agonists of the [Ca2+]i Influx. In the absence of any preperfusion of glutamate, none of the σ drugs alone (i.e., both benzomorphans (+)-SKF-10047 and (−)-pentazocine, JO-1784, NE-100, and DTG) from 0.1 to 20 μM were active on [Ca2+]i influx.

However, after a preperfusion of Glu, 1-min pulses of each (+)-benzomorphan induced an increase of [Ca2+]i, that was not concentration-dependent within the concentration ranges tested. As shown in Figs. 2 and 3, the peak of [Ca2+]i was rapid (within 15 ± 11 s for (+)-SKF-10047 (1 μM) and 12 ± 7 s for (+)-pentazocine (1 μM) from six and three independent experiments, respectively) and lasted less than the (+)-benzomorphan perfusion (mean duration 52 ± 10 s from nine independent experiments). Moreover, both (+)-SKF-10047- and (+)-pentazocine-induced increases of [Ca2+]i were observed exclusively within the 10 min after a Glu-mediated [Ca2+]i, (Figs. 2 and 3), suggesting that an initial neuronal activation is prerequisite for the σ1 agonists to enhance [Ca2+]i. When Ca2+-free buffer was perfused at the end of the first glutamate application, (+)-SKF-10047 (1 μM) failed to increase [Ca2+]i, significantly suggesting that the (+)-benzomorphan facilitated [Ca2+]i influx from the extracellular medium (from three independent experiments).

Conversely to (+)-benzomorphans, JO-1784 (1, 5, and 20 μM) alone never induced an increase of [Ca2+]i after a pulse of glutamate (Fig. 4A; from seven independent experiments).
Similarly, DTG (1 μM; Fig. 5; from six independent experiments) failed to modify [Ca\(^{2+}\)]\(_{i}\) by itself even after a pulse of Glu.

Effects of Benzomorphans on Glutamate-Mediated Increase in [Ca\(^{2+}\)]\(_{i}\). Coperfusion of either (+)-SKF-10047 or (+)-pentazocine with glutamate further enhanced the [Ca\(^{2+}\)]\(_{i}\) increase in response to glutamate alone (Figs. 2 and 3). This enhancement of the [Ca\(^{2+}\)]\(_{i}\) response was maximal from the first coperfusion, exhibiting a concentration dependence that was predominantly apparent with respect to the duration of the [Ca\(^{2+}\)]\(_{i}\) response (mean duration 329 ± 72

versus 165 ± 34 s for glutamate alone, from six independent experiments, respectively, for 1 μM (+)-SKF-10047), the amplitude of the [Ca\(^{2+}\)]\(_{i}\) peak being enhanced by 19 ± 7%.

Coperfusion of glutamate plus (+)-pentazocine (5 μM) resulted in an initial enhancement of 28 ± 13% of the amplitude of the Glu-mediated [Ca\(^{2+}\)]\(_{i}\) increase, whereas the duration of the glutamate response was double. However, repeating 1-min coperfusions resulted in a rapid fading of the potentiating counterpart of the [Ca\(^{2+}\)]\(_{i}\) peak due to the \(\sigma\) agonists already perceptible from the second coperfusion (Figs. 2 and 3). A total desensibilization of the (+)-SKF-10047 (1 μM)-induced potentiation of the glutamate response occurred within 18 ± 3 min (from five independent experi-

Fig. 2. (+)-SKF-10047 initially potentiated glutamate-induced [Ca\(^{2+}\)]\(_{i}\) influx via the \(\sigma\) receptor. A, selective and high-affinity benzomorphans (+)-SKF-10047 (1 μM) was able to induce by itself a [Ca\(^{2+}\)]\(_{i}\) response once glutamate was perfused a few minutes before and to potentiate glutamate (50 μM)-mediated [Ca\(^{2+}\)]\(_{i}\) influx when perfused for the first time with Glu. After several applications of both glutamate and (+)-SKF-10047, the [Ca\(^{2+}\)]\(_{i}\) response returned to baseline. B, concentration-response curve of the initial effect of (+)-SKF-10047 on the glutamate (50 μM)-mediated [Ca\(^{2+}\)]\(_{i}\) influx. C, histograms showing the initial potentiating profile of (+)-SKF-10047 (1 μM) followed by its fading when both glutamate and (+)-SKF-10047 were coapplied at least five consecutive times (values are expressed as \(\Sigma\Delta R/R_0\)). D, whereas KCl (75 mM) enhanced [Ca\(^{2+}\)]\(_{i}\), (+)-SKF-10047 (1 μM) remained ineffective in further potentiating KCl (75 mM)-mediated [Ca\(^{2+}\)]\(_{i}\), influx. Results are from three to six independent cultures and are expressed as mean ± S.E. \(\Sigma\Delta R/R_0\). ** P < 0.01 compared with basal [Ca\(^{2+}\)]\(_{i}\) in the presence of glutamate (50 μM for 1 min) using Student’s \(t\) test.

Fig. 3. (+)-Pentazocine (1 μM) potentiated glutamate (50 μM)-induced [Ca\(^{2+}\)]\(_{i}\) influx via the \(\sigma\) receptor. A, after a pulse of Glu, (+)-pentazocine was able to induce by itself a [Ca\(^{2+}\)]\(_{i}\) response and potentiated glutamate (50 μM)-mediated [Ca\(^{2+}\)]\(_{i}\) influx when perfused for the first time with Glu. After several applications of both glutamate and (+)-pentazocine, the [Ca\(^{2+}\)]\(_{i}\) response returned to baseline. B, concentration-response curve of the initial effect of (+)-pentazocine on the glutamate (50 μM)-mediated [Ca\(^{2+}\)]\(_{i}\), influx. C, histograms showing the initial potentiating profile of (+)-pentazocine (1 μM) followed by its fading when both glutamate and the (+)-benzomorphans were coapplied at least four consecutive times (values are expressed as \(\Sigma\Delta R/R_0\)). Results are from three to five independent cultures and are expressed as mean ± S.E. \(\Sigma\Delta R/R_0\). ** P < 0.01 compared with basal [Ca\(^{2+}\)]\(_{i}\), in the presence of glutamate (50 μM for 1 min) using Student’s \(t\) test.
ments). Higher concentrations (5 and 20 μM) did not modify the latency of the desensitization process (17 and 23 min, respectively). Similarly, a total desensitization of the (+)-pentazocine (1 and 5 μM)-induced potentiation of the glutamate response occurred within 12 ± 7 min (from five independent experiments). Alternation of coperfusions of glutamate plus (+)-SKF-10047 (1 μM) and glutamate plus (+)-pentazocine (1 μM) did not modify this latency (from two independent experiments).

Although cells presented a potentiation of the glutamate response, their profile for the desensitization differed. Once the desensitization of the potentiation had supervened, 6 of 26 hippocampal pyramidal neurons submitted to repeated coperfusions of glutamate plus (+)-SKF-10047 (1 μM) exhibited a 15 to 30% reduction of the evoked [Ca^{2+}] response (from five independent experiments). Similarly, when (+)-pentazocine (5 μM) was used, 5 of 18 recorded neurons exhibited a 35 to 57% reduction of the [Ca^{2+}] influx (from two independent experiments). The remaining neurons exhibited a similar amplitude of the [Ca^{2+}] peak after successive drug applications compared with the pre-α1 agonist perfusion phase.

When KCl (75 mM for 30 s) was used instead of Glu, (+)-SKF-10047 (1 μM) failed to modify the KCl-mediated [Ca^{2+}] increase (Fig. 2C; from two independent experiments).

**Effect of JO-1784 on Glutamate-Mediated Increase in [Ca^{2+}]**

Coperfusion of JO-1784 (0.1, 1, 5, and 10 μM) plus glutamate further enhanced concentration dependently the [Ca^{2+}] influx in response to glutamate alone (Fig. 4; from three to four independent experiments in each condition). This enhancement of the [Ca^{2+}] response was maximal from the first coperfusion, exhibiting a maximal responsiveness at 1 μM concentration. The enhancement concerned both the amplitude (137 ± 8% ΔR/Ro of the glutamate response) and the duration (208 ± 39 versus 149 ± 39 s for glutamate alone; from four independent experiments using μM JO-1784 1) of the [Ca^{2+}] response.

Successive 1-min coperfusions of JO-1784 (1 μM) plus glutamate resulted in a rapid fading of the potentiating counterpart of the [Ca^{2+}] peak due to the α1 agonist that was already perceptible from the second coperfusion (Fig. 4). A total desensibilization of the JO-1784 (1 μM)-induced potentiation of the glutamate response occurred within 12 ± 7 min (from five independent experiments).
tiation of the glutamate response occurred within 13 ± 5 min (from four independent experiments). Once the desensitization occurred, the [Ca^{2+}]_i response to both glutamate and JO-1784 was half reduced (∆ΔR/R_o, 1.4 ± 0.4 versus 3.2 ± 0.4) for all cells.

**Effect of DTG on Glutamate-Mediated Increase in [Ca^{2+}]_i.** DTG (1 μM), known to be active on both σ_1 and σ_2 receptor subtypes (Quirion et al., 1992), reduced the Glu-induced [Ca^{2+}]_i influx in three forth of the pyramidal neurons (24 of 36 recorded neurons exhibiting a decrease of the peak amplitude by 11 ± 3% of the [Ca^{2+}]_i influx, their mean duration being unaffected; from four independent experiments), the other neurons remaining insensitive to the σ drug (Fig. 5). When occurring, the reduction of the Glu-induced [Ca^{2+}]_i response did not fade when 1-min pulses of DTG plus glutamate were repeated. A coperoxification of glutamate plus DTG (1 μM) did not prevent the (+)-SKF-10047 (1 μM)-induced potentiation of the Glu-mediated [Ca^{2+}]_i influx nor modified the latency of the (+)-benzomorphan-induced desensitization (from two independent experiments).

**Effect of NE-100 on the σ_1 Agonist-Induced Modulation of Glutamate-Mediated Increase in [Ca^{2+}]_i.** To ascertain that σ_1 agonists affected the Glu-induced [Ca^{2+}]_i increase via acting at the σ_1 receptor, NE-100, a selective antagonist (Okuyama et al., 1993; Monnet et al., 1996), was used. NE-100 (1 and 5 μM) failed to modify [Ca^{2+}]_i influx even after a pulse of glutamate (Fig. 6A; from four independent experiments). However, at 5 μM, it prevented the potentiation effect of (+)-SKF-10047 (1 μM; Fig. 6C; from four independent experiments) as well as that of (+)-pentazocine (5 μM; from one experiment). When used at a concentration of 1 μM, NE-100 partially reduced the potentiation of [Ca^{2+}]_i influx induced by the association of glutamate plus (+)-SKF-10047 (1 μM; from two independent experiments). It is noteworthy that once the (+)-SKF-10047 (1 μM)-induced potentiation of the Glu-mediated [Ca^{2+}]_i influx had desensitized, unveiling an inconstant inhibitory action of the σ_1 agonist on the Glu-induced [Ca^{2+}]_i response, NE-100 reversed the (+)-SKF-10047-induced reduction of the glutamate response (Fig. 6B; from two independent experiments). Such a block-

![Fig. 6.](image-url) NE-100 failed to modify both glutamate (50 μM)-mediated [Ca^{2+}]_i influx and (+)-SKF-10047 (1 μM)-induced potentiation of the glutamate response. A, selective and high-affinity σ_1 receptor antagonist NE-100 (5 μM) inactive on glutamate-induced [Ca^{2+}]_i influx (A and C) prevented (+)-SKF-10047 (1 μM)-induced modulation of the glutamate response (B and C). After several applications of both glutamate and (+)-SKF-10047, the [Ca^{2+}]_i response returned to baseline. Results are from four independent experiments and are expressed as mean ± S.E. ∆ΔR/R_o; **P < 0.01 compared with basal [Ca^{2+}]_i in the presence of glutamate (50 μM for 1 min) using Student’s t test.

![Fig. 7.](image-url) Go-6976 prevented the potentiation by (+)-SKF-10047 of the glutamate-induced [Ca^{2+}]_i mobilization. The selective protein kinase C inhibitor Go-6976 (200 nM for 30 min before Glu) prevented the (+)-SKF-10047 (1 μM)-mediated potentiation of the glutamate (50 μM)-induced [Ca^{2+}]_i increase, resulting either in an absence of modification of the glutamate response (A) or a reduction of the glutamate response (B). Stimulation of the Ca^{2+}--dependent Fura-2 fluorescence was induced 30 min after the incubation of Go-6976 (200 nM) to neurons by a brief (1 min) bath application of glutamate in the presence of (+)-SKF-10047 (1 μM). Results are from four independent experiments and are expressed as ∆R/R_o.
ade of the effects of (+)-SKF-10047 and (+)-pentazocine by NE-100 is highly suggestive that (+)-benzomorphans act on the \( \sigma_1 \) receptor in the present model.

**Effect of the Prototypic PKC Inhibitor Gö-6976 on the \( \sigma_1 \) Agonist-Induced Modulation of Glutamate-Mediated Increase in [Ca\( ^{2+} \)]\(_i\).** We have previously shown that conventional (c)PKC controls the \( \sigma_1 \) receptor-induced desensitization of neuronal firing activity of hypoglossal motor output (Morin-Surun et al., 1999). Accordingly, to investigate whether cPKC was involved in the hippocampal desensitization process, Gö-6976, which competes at the ATP-binding site of the enzyme thus selectively inhibiting cPKC (Martiny-Baron et al., 1993), was tested. The potentiating effect of (+)-SKF-10047 (1 \( \mu \)M, from four independent experiments) or (+)-pentazocine (5 \( \mu \)M; from two independent experiments) on Glu-induced [Ca\( ^{2+} \)]\(_i\) increase was prevented after Gö-6976 (Fig. 7).

Two different profiles of neuronal responses to coperusions of (+)-benzomorphans plus glutamate were elicited. The first one corresponded to an apparent ineffectiveness of the \( \sigma_1 \) agonist on the glutamate response (24 of 46 neurons recorded with a cosuperfusion of (+)-SKF-10047 (1 \( \mu \)M) plus Glu; from four independent experiments; Fig. 7A). The second one corresponded to an immediate reduction of the Glu-induced [Ca\( ^{2+} \)]\(_i\) increase that lasted as long as the (+)-benzomorphans was applied (22 of 46 neurons recorded from four independent experiments; Fig. 7B). Together, these experiments indicated that cPKC is indeed involved in the \( \sigma_1 \) receptor-mediated modulation of the Glu-induced [Ca\( ^{2+} \)]\(_i\), influx in hippocampal pyramidal neurons.

**Discussion**

The present study shows that the high-affinity \( \sigma_1 \) agonists (+)-SKF-10047, (+)-pentazocine, and JO-1784, but not the mixed \( \sigma_1/\sigma_2 \) agonist DTG, initially potentiated Glu-induced [Ca\( ^{2+} \)]\(_i\), increase from Fura-2-preloaded primary culture of rat hippocampal pyramidal neurons. This effect was sensitive to NE-100 (a selective \( \sigma_1 \) antagonist) and Gö-6976 (a cPKC inhibitor) and rapidly faded, unveiling an inconstant reduction of the glutamate response that was also sensitive to NE-100 but not to Gö-6976.

The blockade of the glutamate response by MK-801 and a Ca\( ^{2+} \)-free buffer is indicative that the NMDA receptor was responsible for the present modulation of [Ca\( ^{2+} \)]\(_i\), consistently with previous reports (Kudo and Ogura, 1986; Hayashi et al., 1995; Limbrick et al., 2001).

Intrinsic action of \( \sigma_1 \) ligands on neuronal activity has not been previously reported for resting hippocampal neurons (Monnet et al., 1990; 1992a,b) but in isolated cardiac myocytes (Novakova et al., 1998) and brainstem hypoglossal neurons (Morin-Surun et al., 1999). Here, (+)-benzomorphans remained ineffective by themselves on [Ca\( ^{2+} \)]\(_i\), unless glutamate has triggered a [Ca\( ^{2+} \)]\(_i\), influx a few minutes before. The fact that this effect was occluded using a Ca\( ^{2+} \)-free buffer indicates that \( \sigma_1 \) receptors can favor [Ca\( ^{2+} \)]\(_i\), influx and is consistent with the above-mentioned studies carried out in cardiac myocytes and brainstem neurons.

The fact that JO-1784 is inactive by itself on [Ca\( ^{2+} \)]\(_i\), (after a pulse of Glu) may support the notion that \( \sigma_1 \) receptors exhibit differential sensitivity to \( \sigma_1 \) agonists (Monnet et al., 1994) and suggests that the molecular mechanism by which (+)-benzomorphans and JO-1784 reduce the glutamate response differs from that responsible for the enhancement of the Glu.

Hayashi et al. (1995) using primary neuronal culture have shown a similar inhibitory effect of DTG on the NMDA receptor-induced [Ca\( ^{2+} \)]\(_i\) influx that we did. DTG also differs from (+)-benzomorphans because it did not modify the desensitization process induced by (+)-SKF-10047. It can be speculated that DTG was acting on \( \sigma_1 \) receptor in the present paradigm, as was the case from SK-N-SH neuroblastoma cells (Novakova et al., 1998; Vilner and Bowen, 2000; Hong and Werling, 2002).

The effectiveness of NE-100 to prevent (+)-benzomorphane-induced modulations of Glu-induced [Ca\( ^{2+} \)]\(_i\), increase as well as the lack of effect of (+)-SKF-10047 on KCl-mediated increase in [Ca\( ^{2+} \)]\(_i\), indicate that the \( \sigma_1 \) receptor per se controls the Glu-induced [Ca\( ^{2+} \)]\(_i\), influx. It is unlikely that the inhibition of the Glu-induced [Ca\( ^{2+} \)]\(_i\), increase by \( \sigma_1 \) drugs correspond to blocking NMDA/PCP receptor properties because the same relatively low concentrations of the \( \sigma_1 \) agonists have initially potentiated the Glu-induced [Ca\( ^{2+} \)]\(_i\), increase.

Finally, the fact that NE-100, devoid of activity on the NMDA/PCP receptor (Okuyama et al., 1993), reverses either the potentiating or the inhibitory effects further support the involvement of the \( \sigma_1 \) receptor.

Molecular mechanisms for neuronal desensitization triggered by (+)-benzomorphans was first described in spontaneously active motor preparation (Morin-Surun et al., 1999) in which the \( \sigma_1 \) receptor translocates from the cytosol to the vicinity of the cell membrane and recruits Ca\( ^{2+} \)-dependent cPKC. Prevention of the induced potentiation by a preadministration of the selective cPKC inhibitor Gö-6976 suggests that the hippocampal \( \sigma_1 \) receptor is functionally linked to cPKC and indicates that it plays a prominent role as neuromodulator and in cell signaling. Novakova et al. (1998) have previously reported a reduced effectiveness of (+)-3-PPP, BD-737, and BD-1047, three high-affinity \( \sigma_1 \) receptor ligands, on [Ca\( ^{2+} \)]\(_i\), transients in isolated cardiac myocytes after successive drug applications, which would be in agreement with our present observation. Finally, these findings, conjugated with those of Hayashi et al. (2000) showing that the \( \sigma_1 \) receptor also control endoplasmic reticulum Ca\( ^{2+} \)-stores, constitute further evidence for a outstanding mode of action of this intracellular receptor for regulating Ca\( ^{2+} \)- dependent signal transduction cascades and neuronal activity.

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**References**


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