Ca\(^{2+}\) Signaling via \(\sigma_1\)-Receptors: Novel Regulatory Mechanism Affecting Intracellular Ca\(^{2+}\) Concentration\(^1\)

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ABSTRACT

The \(\sigma_1\)-receptor is a one-transmembrane endoplasmic reticulum protein that binds neurosteroids and dextrorotatory benzomorphans. The roles of \(\sigma_1\)-receptors in regulating intracellular Ca\(^{2+}\) in NG108 cells were examined in this study. \(\sigma_1\)-Ligands pregnenolone sulfate, (+)-pentazocine, and 2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate hydrochloride modulate Ca\(^{2+}\) signaling in NG108 cells via two modes of action. First, nanomolar concentrations of the ligands, without effect by themselves, potentiated the bradykinin-induced increase of the cytosolic free Ca\(^{2+}\) concentration in a bell-shaped manner. This effect of \(\sigma_1\)-ligands was unaffected by depletion of Ca\(^{2+}\) from perfusion buffer and was blocked by a 21-mer antisense oligodeoxynucleotide against the cloned \(\sigma_1\)-receptors. Second, after the cells were depleted of the endoplasmic reticulum Ca\(^{2+}\) stores, the depolarization (75 mM KCl)-induced increase in cytosolic free Ca\(^{2+}\) was potentiated by 2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate hydrochloride, whereas it was inhibited by pregnenolone sulfate and (+)-pentazocine. These effects, albeit opposite in direction, were blocked by both the 21-mer antisense oligodeoxynucleotide and pertussis toxin. Western blotting indicates that \(\sigma_1\)-receptors are increased on the plasma membrane and the nuclear membrane in the presence of \(\sigma_1\)-ligand. These results suggest that Ca\(^{2+}\) signaling via \(\sigma_1\)-receptors may represent a novel mechanism that affects intracellular Ca\(^{2+}\) concentrations.

\(\sigma_1\)-Receptors are a subtype of "\(\sigma\)-receptors" known to bind diverse classes of pharmacological agents with high affinity. These agents include neurosteroids, antipsychotics, and dextrorotatory benzomorphans (Snyder and Largent, 1989; Su, 1991). \(\sigma\)-Receptors are not the "\(\sigma\)opioid" receptors originally proposed by Martin et al. (1976) to mediate psychotomimetic effects of benzomorphans. \(\sigma_1\)-Receptors are currently thought to be involved, among other functions, in learning and memory as well as in the analgesic processes of animals (Chien and Pasternak, 1994; Maurice et al., 1994, 1998; Bouchard et al., 1997).

The \(\sigma_1\)-receptor has been cloned (Hanner et al., 1996; Prasad et al., 1998). The deduced amino acid sequence does not resemble that of any mammalian protein. The sequence of \(\sigma_1\)-receptors contains an endoplasmic reticulum (ER) retention signal close to the N terminus, a binding domain for steroid, and one putative transmembrane region. Although the cloned \(\sigma_1\)-receptor has a 30% identity and a 60% homology to a yeast C7-C8 sterol isomerase, \(\sigma_1\)-receptors apparently possess no similar enzymatic activity. Furthermore, the mammalian equivalent of the C7-C8 sterol isomerase has been cloned and the deduced amino acid sequence is different from that of both the yeast C7-C8 sterol isomerase and the \(\sigma_1\)-receptor (Silve et al., 1996). Thus, although the structure of \(\sigma_1\)-receptors is known, the biochemical basis subserving the action of \(\sigma_1\)-receptors remains elusive.

Several lines of evidences have suggested that \(\sigma\)-receptors may be related to the regulation of intracellular Ca\(^{2+}\). For example, exposure of cardiac myocytes to \(\sigma\)-ligands was found to affect contractility, Ca\(^{2+}\) influx, and beating rate (Ela et al., 1994) and to increase intracellular level of inositol 1,4,5-trisphosphate (IP\(_3\)) in cultured myocytes (Novakova et al., 1998). \(\sigma\)-Ligands affect intrasynaptosomal free Ca\(^{2+}\) levels in rat forebrain synaptosomes and protein phosphorylation (Brent et al., 1997). Certain \(\sigma\)-ligands also have been

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ABBREVIATIONS: ER, endoplasmic reticulum; IP\(_3\), inositol 1,4,5-trisphosphate; NMDA, N-methyl-D-aspartate; NE-100, N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride; AS ODN, antisense oligodeoxynucleotide; PS, pregnenolone sulfate; (+)-PTZ, (+)-pentazocine; PRE-084; 2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate hydrochloride; PT, pertussis toxin; BDK, bradykinin; TG, thapsigargin; HBSS, Hanks’ balanced salt solution; MS ODN, mismatched oligodeoxynucleotide; TBST, Tris-buffered saline/Tween 20; Prog, progesterone; GABA, \(\gamma\)-aminobutyric acid; T + B + C, TG + BDK + caffeine; VDCC, voltage-dependent Ca\(^{2+}\) channel.
shown to affect N-methyl-d-aspartate (NMDA)-induced Ca\(^{2+}\) signaling in rat primary neurons (Hayashi et al., 1995; Klette et al., 1997). However, none of these studies has definitively demonstrated a clear-cut agonist-antagonist relationship in which pharmacologically relevant concentrations of ligands were used and the antagonists by themselves produced no effect. Furthermore, none of the above-mentioned studies has definitively demonstrated which subtype of \(\sigma\)-receptors (i.e., \(\sigma_1\) or \(\sigma_2\); Quirion et al., 1992; Hellewell et al., 1994) mediated these effects.

With the availability of a selective \(\sigma_1\)-receptor antagonist \(N,N\)-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) (Okuyama and Nakanzato, 1996) and the successful demonstration of the use of an antiserum against the cloned \(\sigma\)-ligand of different chemical class, were examined.

### Experimental Procedures

**Materials.** PS, pertussis toxin (PT), MK-801 (dizocilpine), (+)-bicuculline, and bradykinin (BDK) were purchased from Research Biochemicals International (Natick, MA); Thapsigargin (TG), Tween 20, phenylmethylsulfonyl fluoride, and aprotinin were from Calbiochem (San Diego, CA). (+)-PTZ was obtained from Research Triangle Institute (Research Triangle Park, NC). [\(^{3}H\)IP3 and the IP3 assay kit were purchased from Amersham (Arlington Heights, IL). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

**Confocal Fluorescence Microscopic Examination.** NG108 cells were cultured as described before (Hescheler et al., 1987) and maintained on petriPERM with a four-well FlexiPERM (Heraeus Instr., Am Kalkberg, Germany) attached to it. The cultured cells on petriPERM were washed with Hanks’ balanced salt solution (HBSS) (137 mM NaCl, 0.4 mM MgSO\(_4\), 0.5 mM MgCl\(_2\), 5.4 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 0.3 mM Na\(_2\)HPO\(_4\), 3.0 mM NaHCO\(_3\), 1.5 mM CaCl\(_2\), 5.6 mM glucose, and 20 mM HEPE, pH 7.4) and loaded with 5 \(\mu\)M fluo-3/AM (Molecular Probes, Eugene, OR) in HBSS for 25 min at room temperature. Then uniform fluorescence intensities were observed in each cell. Permeabilization of cells with 10 \(\mu\)M digitonin in a Ca\(^{2+}\)-free medium completely eliminated the fluorescence signals inside the cells, indicating that the observed fluorescence intensities represent free Ca\(^{2+}\) in the cytosol (Golovina and Blaustein, 1997). After loading, cells were placed on the microscope stage and perfused continuously (2 ml/min) with HBSS at 37°C by using two peristaltic pumps (input and output) to maintain the test well volume at \(-200 \mu\)l. One end of the solution input line was attached to a plastic pipette tip that was placed 1.5 mm above the cells. The other end of the input line was placed into beakers containing HBSS or HBSS with drugs at test concentrations. In the BDK experiments, HBSS containing \(\sigma\)-ligands was perfused into the well for at least 10 min. Afterward, a solution containing both the \(\sigma\)-ligands and 1 \(\mu\)M BDK was perfused into the well for additional 2 min. In the KCl experiments, the cells were first perfused with the HBSS containing a test drug (such as \(\sigma\)-ligands) or a combination of several different drugs for 10 min. The same solution with an addition of 75 mM KCl was then applied to the cells for a duration of 30 s before the solution was replaced with the original solution containing only the test ligand(s). In this perfusion system, maximal response to KCl was reached within 3 to 5 s. Dye-loaded cells were perfused with 37°C HBSS and the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)] was monitored in each cell according to the formula:

\[
F(t) = F_{max} - F_{bgk} \frac{(F_{max} - F_{bgk})/40}{F_{max} - F_{bgk}} = K_d (F(t) - F_{min})/(F_{max} - F(t))
\]

where \(K_d\) equals \((F_{max} - F_{bgk})/40\), and \(F_{max}\) is the average of fluorescence intensities for 30 s with ionomycin (10 \(\mu\)M) and MnCl\(_2\) (2 mM), and \(F_{bgk}\) is the average of fluorescence intensities for 30 s with ionomycin (10 \(\mu\)M) and MnCl\(_2\) (2 mM) after lysis of cells with 40 \(\mu\)M digitonin. In this report, each experimental determination used a four-well FlexiPERM plate. An average of only three to nine cells per culture well, but never more than nine cells per well, was examined in each determination, which always included a control well. In each determination, a similar treatment condition was never repeated in other wells except occasionally the controls were repeated. Statistical significance was analyzed by comparing values from all cells receiving the same treatment condition in separate determinations. In this report, the number of determinations in each experimental group is indicated in parentheses after the number of cells examined.

**AS ODN Transfection.** Genes of the \(\sigma_1\)-receptor were highly conserved from mouse to humans (Hanner et al., 1996; Prasad et al., 1998). Therefore, a 21-mer AS ODN, which was published based on the mouse sequence for \(\sigma_1\)-receptor (King et al., 1997), was synthesized with the Cyclone Plus DNA synthesizer (Milli Gen/biosearch, Bedford, MA), purified through an NAP-10 column (Pharmacia Biotech, Uppsala, Sweden), and used in this study. The sequences of the AS ODN and the mismatched oligodeoxynucleotide (MS ODN) control with three pairs of nucleotide substitutions were, respectively (King et al., 1997): 5’-GAGTTCCGAGCCACAGG-3’ and 5’-GAGTTCCGAGCCACAGG-3’.

When NG108 cells reached a 40 to 50% confluent, adherent cells were washed with culture medium containing reduced fetal calf serum (5%) without penicillin and streptomycin. For transfection, cells were treated with 200 \(\mu\)l of the same medium containing Lipofectin reagent (Life Technologies, Gaithersburg MD) and the AS ODN or MS ODN at 3.75 \(\mu\)g/ml. Cells were incubated at 37°C under 10% CO\(_2\) for 6 h and then 0.8 ml of Dulbeco’s modified Eagle’s medium with 10% fetal calf serum (without antibiotics) was added to each culture well. Cells were used for experiments after 48 to 72 h after the cell transfection. The culture medium for the transfection experiment contains 0.1 mM hypoxanthine, 400 nM aminopterin, and 16 \(\mu\)M thymidine.
Determination of IP$_3$ Concentration. For the determination of contents of IP$_3$, NG108 cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, and 1.4 mM KH$_2$PO$_4$, pH 7.4) and resuspended in HBSS containing 10 mM LiCl at a density of $2 \times 10^5$ cells/ml. Cells ($6 \times 10^4$ in 300-μl aliquots were precultured for 10 min at 37°C and then stimulated with different concentrations of BDK, in the presence and absence of 100 nM (+)-PTZ, for 15 s. The reaction was terminated by adding 0.2 volumes of ice-cold 20% perchloric acid. The resultant samples were adjusted to pH 7.5 with KOH-HEPES buffer. Neutralized samples were centrifuged to remove KClO$_4$, and the supernatant was used for the IP$_3$ assay in a radioligand method with $[^3H]$IP$_3$ as previously described in Katayama et al. (1994).

Immunodetection of $\sigma_1$-Receptors by Western Blotting. A polyclonal antibody was raised in rabbit against a 20-amino acid peptide corresponding to the fragment 143 to 162 (synthesized by Neostrsys, Strasbourg, France) of rat $\sigma_1$-receptor. The peptide was conjugated to BSA with glutaraldehyde (1 mg/peptide/5 mg of BSA) and the complex was dialyzed against saline. Three rabbits were subsequently subjected to a standard boost of the complex peptide with Freund's adjuvant. For each rabbit, serum was collected before immunization and every 6 weeks after the third injection. The different sera obtained were screened by immunostaining with forebrain sections obtained from adult rats. The antisera giving the most intense immunostaining was selected and purified against the antigenic peptide with the HiTrap N-hydroxysuccinimide-activated affinity column (Pharmacia Biotech). The immunostaining was reduced by the antigen peptide. NG108 cells were washed and incubated with HBSS for 30 min. Cells were treated with or without $\sigma_1$-ligands. The membrane fractions from NG108 cells and rat hippocampus to be used for Western blotting were prepared as described previously and P1, P2, and P3 fractions were obtained accordingly (Furuichi et al., 1989; Miyawaki et al., 1991). Thirty micrograms of protein was dissolved in SDS gel sample buffer and separated by 12% SDS-polyacrylamide gel electrophoresis. For Western blotting, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA). The nonspecific sites were blocked with 5% (w/v) nonfat dry milk (Bio-Rad) in Tris-buffered saline/Tween 20 (TBST; 10 mM Tris-HCl, pH 8.0; 150 mM NaCl, and 0.05% Tween 20) at 4°C overnight. For immunodetection of $\sigma_1$-receptor, antibody to $\sigma_1$-ligands was diluted 2000-fold in TBST containing 2% milk and incubated with membrane at 30°C for 2 h. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 dilution; 1 h at room temperature; Amersham) in TBST incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 dilution; 1 h at room temperature; Amersham) in TBST, washed and visualized by using an enhanced chemiluminescence detection kit (Amersham).

Statistical Analyses. ANOVA was first used to check the statistical tendency of experimental data. Differences between groups of data were then examined post hoc by Fisher’s protected least-significant difference test or Student’s t test. All data presented represent mean ± S.E. The significance level was set at P < .05.

Results

The first experiment tested the effects of $\sigma_1$-ligands PS, (+)-PTZ, and PRE-084 on the basal level of [Ca$^{2+}$]$_{cyt}$ in NG108 cells. These three ligands when tested alone under the concentrations used in this study did not cause any change on the basal [Ca$^{2+}$]$_{cyt}$. We speculated that $\sigma_1$-receptors via which these ligands act may play a modulatory role in the present system. The speculation was not far-fetched. For example, $\sigma$-ligands have been shown to exhibit no or minimal effects by themselves in affecting phosphoinositide turnover whereas are able to modulate carbachol-induced (i.e., stimulated) phosphoinositide turnover in brain synaptoneurosomes (Bowen et al., 1988). Also, consistent with this speculation, in neurophysiological studies, $\sigma$-ligands have been shown to cause no effects of their own unless compound such as NMDA or neuropeptide Y was added to perturb the system (Monnet et al., 1992). We decided therefore to test the activities of PS, (+)-PTZ, and PRE-084 in systems that might represent a perturbed state of [Ca$^{2+}$]$_{cyt}$ and not that of a resting condition. Two systems were chosen. The first examined the effects of these $\sigma_1$-ligands on the BDK-induced increase of [Ca$^{2+}$]$_{cyt}$. The second examined the effects of these ligands on the depolarization-induced increase in [Ca$^{2+}$]$_{cyt}$.

We first tested the effects of $\sigma_1$-ligands on the increase of [Ca$^{2+}$]$_{cyt}$ induced by BDK. BDK is known to, via BDK receptors, increase [Ca$^{2+}$]$_{cyt}$ concentration by increasing the formation of intracellular IP$_3$, which in turn increases the [Ca$^{2+}$]$_{cyt}$ via IP$_3$ receptors on the ER (Berridge, 1993; Thomas et al., 1996). In this study, BDK was found to concentration dependently cause a sharp rise in [Ca$^{2+}$]$_{cyt}$ in NG108 cells, which subsided within 20 s (Fig. 1, inset). The peak height of [Ca$^{2+}$]$_{cyt}$ was used for comparison of effects caused by different ligands in this study. Typically, 1 μM BDK increased the [Ca$^{2+}$]$_{cyt}$ from a basal level of 78.3 ± 5.4 to 889 ± 84 nM (mean ± S.E.; n = 24 cells). Pretreatment of cells with PT abolished the effect caused by BDK (data not shown). BDK-induced [Ca$^{2+}$]$_{cyt}$ response was quantified by subtraction of the resting level from maximal [Ca$^{2+}$]$_{cyt}$ elicited by BDK. This effect by BDK is an intracellular event because the removal of Ca$^{2+}$ from the perfusion buffer did not significantly affect the effect caused by BDK (Fig. 1, upper right inset). The Ca$^{2+}$ chelator could not be used in the experiment because the chelator caused cells to detach from culture plates. Also, the TG treatment suppressed the action of BDK. The results are in alignment with the established notion that BDK increases [Ca$^{2+}$]$_{cyt}$ by mobilizing Ca$^{2+}$ from intracellular stores (Berridge, 1993). PS (0.1–100 μM), (+)-PTZ (0.001–10 μM), and PRE-084 (0.01–10 μM) concentration dependently potentiated the increase of [Ca$^{2+}$]$_{cyt}$ caused by BDK in a bell-shaped manner with the peak height at ~1, 0.1, and 0.1 μM, respectively (Fig. 1). However, it has to be mentioned that these potentiating effects exerted by $\sigma$-ligands can only be seen after a close to 10-min incubation of cells with the $\sigma$-ligands. The 10-min incubation was a minimal effective period. Treatment of cells with $\sigma_1$-ligands for <10 min did not produce a significantly different effect. Progesterone (Prog; 0.001–10 μM), another $\sigma$-ligand (Su et al., 1988), also was tested. Prog at <1 μM tended to potentiate the BDK effect. However, this effect of Prog was not statistically significant. Prog at 10 μM tended to decrease the BDK effect (Fig. 1, right). A selective $\sigma_1$-antagonist NE-100 (0.001–1 μM) by itself produced no effect on that caused by BDK (Fig. 1, right).

The potentiating action of PS on the BDK-induced increase in [Ca$^{2+}$]$_{cyt}$ was not affected by either the NMDA receptor channel blocker MK-801 or the γ-aminobutyric acid (GABA)$_\lambda$ receptor antagonist (+)-bicuculline (Fig. 2a), but was antagonized by a selective $\sigma_1$-receptor antagonist NE-100 (1 μM; Fig. 2a). NG108 cells have been shown to contain NMDA receptors (Ohkuma et al., 1994) as well as GABAA receptors (Yoshii et al., 1997). These results indicate that PS potentiated the [Ca$^{2+}$]$_{cyt}$ increase induced by BDK via $\sigma_1$-receptors and not via NMDA or GABA$_\lambda$ receptor. It was noted that MK-801 slightly enhanced, but not to a statistically significant level, the BDK-induced [Ca$^{2+}$]$_{cyt}$ increase either in the absence or presence of PS (Fig. 2a). No report in the litera-
ANOVA was used to analyze each curve. Fisher’s protected least-significant difference test was used post hoc to examine differences between groups.

Cloned the presence of 1 increase of the BDK effect (Fig. 2b, center). Nevertheless, in the same treatment in multiple determinations (see Experimental Procedures for details). The number of determinations per experimental group is indicated in parentheses after the cell numbers in this report. Representative traces of the BDK-induced increase in [Ca^{2+}]_{cyt} in the presence [PS(+); 1 µM] and absence [PS(-)] of PS are shown in the inset. The other curves represent the effects of α-ligands in potentiating the BDK-induced increase in [Ca^{2+}]_{cyt}. Data are based on a 10-min pretreatment time of cells with PS [n = 37–62 cells (8–10 per dose)], (+)-PTZ [n = 31–74 cells (7–12 per dose)], PRE-084 [n = 2–91 cells (6–14 per dose)], progesterone [Prog; n = 20–36 cells (5–7 per dose)], and NE-100 [n = 23–46 cells (5–8 per dose)]. One-way ANOVA was used to analyze each curve. Fisher’s protected least-significant difference test was used post hoc to examine differences between groups of data. *P < 0.05, **P < 0.01 compared with BDK alone without the α-ligands. Upper right inset, removal of Ca^{2+} from perfusion medium did not significantly affect the BDK-induced increase in [Ca^{2+}]_{cyt} [n = 51 and 39 cells (9 and 6), respectively, with and without Ca^{2+}; P = 0.889].

Fig. 1. Potentiation of BDK-induced increase in [Ca^{2+}]_{cyt} in NG108 cells by PS and other nonsteroidal α-receptor ligands (+)-PTZ and PRE-084 (PRE). An average of three to nine cells from each culture well were examined per determination. Data represent mean ± S.E. of values from all cells receiving the same treatment in multiple determinations (see Experimental Procedures for details). The number of determinations per experimental group is indicated in parentheses after the cell numbers in this report. Representative traces of the BDK-induced increase in [Ca^{2+}]_{cyt} in the presence [PS(+); 1 µM] and absence [PS(-)] of PS are shown in the inset. The other curves represent the effects of α-ligands in potentiating the BDK-induced increase in [Ca^{2+}]_{cyt}. Data are based on a 10-min pretreatment time of cells with PS [n = 37–62 cells (8–10 per dose)], (+)-PTZ [n = 31–74 cells (7–12 per dose)], PRE-084 [n = 2–91 cells (6–14 per dose)], progesterone [Prog; n = 20–36 cells (5–7 per dose)], and NE-100 [n = 23–46 cells (5–8 per dose)]. One-way ANOVA was used to analyze each curve. Fisher’s protected least-significant difference test was used post hoc to examine differences between groups of data. *P < 0.05, **P < 0.01 compared with BDK alone without the α-ligands. Upper right inset, removal of Ca^{2+} from perfusion medium did not significantly affect the BDK-induced increase in [Ca^{2+}]_{cyt} [n = 51 and 39 cells (9 and 6), respectively, with and without Ca^{2+}; P = 0.889].

Removal of Ca^{2+} from perfusion buffer did not affect the potentiating action of (+)-PTZ on the BDK-induced increase in [Ca^{2+}]_{cyt} (data not shown). This indicates that α-ligands potentiate the BDK-induced increase of [Ca^{2+}]_{cyt} through an intracellular site of action. Because BDK is known to increase [Ca^{2+}]_{cyt} by increasing the formation of intracellular IP_{3}, we examined whether the intracellular site of action exerted by α-ligands might be related to their ability to potentiate the formation of IP_{3} induced by BDK. Therefore, IP_{3} concentrations were measured. In the absence of BDK, (+)-PTZ (100 nM) did not alter the basal concentration of IP_{3} over the observation period of 10 min (Fig. 3a). BDK, as expected, increased the IP_{3} formation in a concentration-dependent manner (Fig. 3b). (+)-PTZ, however, attenuated the maximal ceiling effect exerted by BDK in IP_{3} formation (Fig. 3b). These results suggest that α-ligands affect BDK-induced [Ca^{2+}]_{cyt} intracellularly at a locus beyond the IP_{3} formation. Because IP_{3} increases [Ca^{2+}]_{cyt} by acting on the IP_{3} receptors on the ER where α-receptors are abundant, it is possible that α-ligands may affect [Ca^{2+}]_{cyt} by potentiating the ac-
Fig. 2. Antagonism of the potentiating effect of $\sigma$-ligands on BDK-induced increase in $[Ca^{2+}]_{cyt}$ by various drugs or ODNs directed against $\sigma_1$-receptors. a, effect of PS (1 $\mu$M) by MK-801 (10 $\mu$M), (+)-bicuculline (100 $\mu$M), and NE-100 (1 $\mu$M) [$n = 40–55$ cells (6–8) per group]. BDK was present in all experiments. Student's $t$ test was used to examine differences between each control (i.e., BDK + antagonist alone) and the antagonist-treated (i.e., BDK + antagonist + PS) group. *$P < .05$ compared with respective controls. b, effects of (+)-PTZ (100 nM) and PRE-084 (100 nM) by NE-100 (1 $\mu$M; $n = 48–55$ cells (6–8) against (+)-PTZ; $n = 34–66$ cells (5–7) against PRE] and Prog [1 $\mu$M; $n = 28–33$ cells (5–6) against
tion of IP₃ on the ER via an as-yet-unknown mechanism. These results also suggest that the descending phase of the bell-shaped dose-response curves seen in Fig. 1 may be due to the σ₁-ligand suppressing the IP₃ formation at high doses.

As mentioned in the Introduction, α-receptors are known to exist not only on the ER but also on the plasma membrane with the latter containing less α-receptors (McCann and Su, 1990). As such, we examined whether σ₁-receptors might regulate the [Ca²⁺]ₜₖᵢₙ increase caused by KCl-induced depolarization in NG108 cells. However, to examine any potential action of σ₁-receptors on the plasma membrane, it was necessary to eliminate [Ca²⁺]ₜₖᵢₙ changes attributable to the intracellular sites of action caused by σ₁-ligands. KCl (75 mM) induced depolarization in NG108 cells. The components of the apparent increase of [Ca²⁺]ₜₖᵢₙ caused by the KCl-induced depolarization were characterized. KCl (75 mM) evoked an initial transient increase in [Ca²⁺]ₜₖᵢₙ, appearing as an apparent peak height, followed by a declined phase that subsided when KCl was withdrawn. Typically, KCl (75 mM) increased [Ca²⁺]ₜₖᵢₙ from an average peak height of 72.3 ± 5.6 to 292.8 ± 11.1 nM (n = 42 cells from nine determinations). In the absence of extracellular Ca²⁺, KCl-induced increase in [Ca²⁺]ₜₖᵢₙ was almost totally diminished (11.4 ± 2.2%; n = 13 cells from three determinations) compared with controls with normal extracellular Ca²⁺ in the perfusion buffer (Fig. 4, filled columns). Nifedipine, an L-type Ca²⁺ channel blocker, dose dependently inhibited the KCl-induced increase in [Ca²⁺]ₜₖᵢₙ. Nifedipine at 100 nM attenuated the KCl-induced increase in [Ca²⁺]ₜₖᵢₙ to only 15.1 ± 4.2% of that induced by 75 mM KCl without nifedipine (n = 10 cells from three determinations; Fig. 4, filled columns). The IC₅₀ of nifedipine in inhibiting the KCl-induced effect was ~25 nM. These results indicate that the 75 mM KCl-induced increase of [Ca²⁺]ₜₖᵢₙ involved the voltage-dependent L-type Ca²⁺ channel (VDCC). Because the apparent increase of [Ca²⁺]ₜₖᵢₙ induced by KCl depolarization also might involve certain components attributable from mobilization of Ca²⁺ from intracellular Ca²⁺ stores, including the "Ca²⁺-induced Ca²⁺ release" (Berridge, 1993; Thomas et al., 1996), we examined the effects of compounds that are known to affect intracellular Ca²⁺ mobilization in the KCl-treated cells. The pretreatment of cells for 10 min with TG (1 μM), an inhibitor of Ca²⁺-ATPase on the ER (Gill et al., 1996), attenuated the KCl-induced increase in [Ca²⁺]ₜₖᵢₙ to 62.0 ± 2.7% of control; n = 12 cells from three determinations; Fig. 4, unfilled columns). BDK (300 nM) did not affect the KCl-induced [Ca²⁺]ₜₖᵢₙ if administered alone to cells for 10 min before the KCl challenge. However, administration to cells with a combination of BDK (300 nM) plus TG (1 μM) for 10 min could further attenuate the KCl-induced increase in [Ca²⁺]ₜₖᵢₙ compared with TG pretreatment alone (to 49.4 ± 2.7% of controls; n = 12 cells from three determinations; Fig. 4, unfilled columns). Similarly, the addition of caffeine (3 mM), a ryanodine receptor agonist (Gill et al., 1996), to the pretreatment solution containing TG and BDK also caused a further attenuation of KCl-induced increase in [Ca²⁺]ₜₖᵢₙ compared with the TG pretreatment alone (to 39.3 ± 1.9% of controls; n = 12 cells from three determinations; Fig. 4, unfilled columns). These data, when taken together with the above-mentioned results with nifedipine, indicate that the 39% of KCl-induced increase in [Ca²⁺]ₜₖᵢₙ in NG108 cells apparently arose from Ca²⁺ influx across the plasma membrane via VDCC, and the rest of the 61% came from Ca²⁺ released from intracellular stores as a result of the Ca²⁺ influx at the plasma membrane, including Ca²⁺-induced Ca²⁺ release (Berridge, 1993; Thomas et al., 1996). It has to be mentioned herein that caffeine was found in a report to cause an influx of Ca²⁺ from the extracellular space that lasted for at least 3 min when tested in TG-pretreated DDT1

(+)-PTZ] treatments. Data from each group (i.e., per four columns) was analyzed by one-way ANOVA followed by Fisher’s protected least-significant difference test. Each group has its own control (○). *P < .05, **P < .01 compared with controls. c, effects of PS and (+)-PTZ by antisense (AS) ODN directed against α₁-receptors. See Experimental Procedures for ODN transfection. Forty-eight hours after the transfection, cells were subjected to experiments. MS ODN was used in the experiment for comparison (n = 29–39 cells (5–7 per group). Student’s t test was used to compare differences between each column and the control (○). *P < .05, **P < .01 compared with control.
MF-2 cells (Gill et al., 1996). However, in the present study, we found that, during the 10-min pretreatment with a combination of TG plus caffeine, the overall [Ca\(^{2+}\)]\(_{cyt}\) profile was the same as that with TG pretreatment alone, except that an initial transient rise in [Ca\(^{2+}\)]\(_{cyt}\) was observed that was attributable to caffeine. This suggests an absence of caffeine-induced Ca\(^{2+}\) influx similar to that reported by Gill et al. (1996) in TG-pretreated NG108 cells. The reason for the discrepancy is unknown at present. Nevertheless, collectively, our results indicate that to study the potential role of \(\alpha_1\)-receptors on the modulation of Ca\(^{2+}\) influx across the plasma membrane, a cocktail of 1 mM TG, 300 nM BDK, and 3 mM caffeine for a duration of time (10 min) can be used to nearly deplete the ER Ca\(^{2+}\) stores, leaving the majority of [Ca\(^{2+}\)]\(_{cyt}\), reflecting an increase sequel to a Ca\(^{2+}\) influx from the extracellular space. Cells were thus treated with a cocktail containing 1 \(\mu\)M TG, 300 nM BDK, and 3 mM caffeine before use. The combination of the three drugs is abbreviated as TG + B + C and used in the following studies to ensure that the effects seen with \(\alpha_1\)-ligands were mediated via an action on the plasma membrane.

After the treatment of TG + B + C, 75 mM KCl caused a depolarization in NG108 cells and an increase in [Ca\(^{2+}\)]\(_{cyt}\). Again, under the basal condition (i.e., without KCl) in the presence of TG + B + C, PS, (+)-PTZ, and PRE-084 caused no effect of their own. However, PS and (+)-PTZ concentration dependently inhibited the KCl-induced increase in [Ca\(^{2+}\)]\(_{cyt}\), whereas PRE-084 potentiated the KCl-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) (Fig. 5a). Similar to the BDK studies presented earlier, it required a 10-min incubation of cells with the \(\alpha_1\)-ligands to see these effects caused by the ligands. However, unlike the BDK studies (Fig. 1), the dose-response curves seen herein are regular and not bell shaped. These results, when taken together with results obtained in Fig. 3, suggest an absence of the involvement of IP\(_3\) receptors in the effects of \(\alpha_1\)-ligands in affecting the depolarization-induced Ca\(^{2+}\) influx at the plasma membrane.

The 21-mer AS ODN (King et al., 1997) and the corresponding MS ODN (King et al., 1997) designed to act against cloned \(\alpha_1\)-receptors were again used to examine whether these effects caused by \(\alpha_1\)-ligands, albeit being inhibitory or potentiating, were mediated by \(\alpha_1\)-receptors. The AS ODN treatment significantly blocked both the inhibitory effect caused by (+)-PTZ (Fig. 5b) and the potentiating effect caused by PRE-084 (Fig. 5b; ANOVA and Student’s t tests). For the action of PS, the AS ODN treatment, although it did not significantly block the effect produced by PS when data were compared at each individual test dose of PS (Student’s t test; Fig. 5b), could however significantly abolish the overall dose-dependent effect caused by PS (ANOVA). Thus, PS produced no significant overall effect when cells were pretreated with AS ODN (Fig. 5b). The MS ODN has no effect at all on all these actions caused by \(\alpha_1\)-ligands (Fig. 5b).

Because the action of \(\alpha_1\)-receptors have been suggested to be related to G protein (Monnet et al., 1992, 1994), we examined if these actions by \(\alpha_1\)-ligands on the plasma membrane in this study are sensitive to PT treatment. Results showed that PT treatment totally abolished the inhibitory action caused by (+)-PTZ. The potentiating effect caused by PRE-084 on the depolarization-induced increase of [Ca\(^{2+}\)]\(_{cyt}\) was diminished to a nonsignificant level (Fig. 6; see legend).

Immunoblotting analyses with the polyclonal antibodies raised against fragment 143 to 162 of rat \(\alpha_1\)-receptor for 24 h attenuated the level of immunoreactive peptide close to that of the cloned \(\alpha_1\)-receptor (Hanner et al., 1996). In NG108 cells without an incubation with \(\alpha_1\)-ligand, the immunoreactive \(\alpha_1\)-receptors were predominantly present in microsomes (P3), barely detectable in the plasma membrane (P2) and the nuclear membrane (P1), and not detectable in the cytosol (Fig. 7b, top). However, when the NG108 cells were treated with (+)-PTZ (100 nM) for 10 min, relative intensity of immunoreactive \(\alpha_1\)-receptors increased both in the plasma membrane and the nuclear membrane (Fig. 7b, bottom). Treatment of NG108 cells with the 21 mer AS ODN directed against the cloned \(\alpha_1\)-receptor for 24 h attenuated the level of immunoreactive \(\alpha_1\)-receptors (Fig. 7c; AS1). Forty-eight hours after the AS ODN treatment, the level of \(\alpha_1\)-receptors was significantly reduced (Fig. 7c; AS2). The MS ODN did not affect the level of \(\alpha_1\)-receptors (Fig. 7c; MS).
Discussion

We have clearly demonstrated that, in nanomolar concentrations, the endogenous neurosteroid PS and the other α-ligands (+)-PTZ and PRE-084 can modulate Ca\(^{2+}\) signaling via α\(_1\)-receptors in two different modes of action: one an intracellular action perhaps on the ER, and the other on the plasma membrane via a G-protein-dependent action. α\(_1\)-Receptors may thus represent a newly recognized, unique one-transmembrane protein regulating Ca\(^{2+}\) signaling in cells.

This study constitutes the first report demonstrating a clear-cut agonist-antagonist relationship for α\(_1\)-receptors with ligands being effective in nanomolar concentrations and antagonist showing no effect of its own in a simple biological system such as an established cell line. Our results with PS also represent the first direct demonstration that endogenous neurosteroids can affect Ca\(^{2+}\) signaling via the newly recognized α\(_1\)-receptor system in cells.

Our results showing that the potentiating effects of α\(_1\)-ligands on the BDK-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) was not affected by the removal of Ca\(^{2+}\) from perfusion medium suggest that the loci of the action of these ligands are intracellular and are perhaps on the ER because α\(_1\)-receptors are ER proteins and the BDK effect was blocked by the AS ODN against α\(_1\)-receptors. Further substantiating this notion is our results showing that the IP\(_3\) concentration was not increased by α-ligands. In fact, the α-ligand tended to decrease the efficacy of the BDK-induced increase of IP\(_3\) formation (Fig. 3). These results are in contrast to the report that showed that α-ligands increased the basal level of IP\(_3\) in cultured myocytes (Novakova et al., 1998). The discrepancy...
The overall PRE-084 effect and (−)-PTZ effect were not significant in the plasma membrane and nuclear membrane in the presence of T + B + C (see Experimental Procedures). Cells were treated with 50 ng/ml PT in culture medium for 18 h before experiments. KCl (75 mM) was used to induce depolarization [n = 40–45 cells (6–8) at each dose of test drug]. Each curve was analyzed by one-way ANOVA followed by Fisher’s protected least-significant difference test as a post hoc test for examining statistical differences within a curve. The difference between two data points of different curves, at the same concentration of test drug, was analyzed by Student’s t test. *P < .05, **P < .01 compared with controls. The overall PRE-084 effect and (−)-PTZ effect were not significant in the presence of PT (ANOVA: P = .25 and P = .79, respectively). *P < .01 between cells with PT [PT(+) and without PT treatment [PT(−)].

Fig. 6. Effect of PT pretreatment on the modulation of depolarization-induced [Ca2+]cyt increase by α-ligands in NG108 cells that were depleted of ER Ca2+ stores. Depletion of ER Ca2+ stores was achieved by using T + B + C (see Experimental Procedures). Cells were treated with 50 ng/ml PT in culture medium for 18 h before experiments. KCl (75 mM) was used to induce depolarization [n = 40–45 cells (6–8) at each dose of test drug]. Each curve was analyzed by one-way ANOVA followed by Fisher’s protected least-significant difference test as a post hoc test for examining statistical differences within a curve. The difference between two data points of different curves, at the same concentration of test drug, was analyzed by Student’s t test. *P < .05, **P < .01 compared with controls. The overall PRE-084 effect and (−)-PTZ effect were not significant in the presence of PT (ANOVA: P = .25 and P = .79, respectively). *P < .01 between cells with PT [PT(+) and without PT treatment [PT(−)].

Fig. 7. Western blotting of α1-receptors: effects of the α-ligand treatment and the AS ODN treatment. The antibody was raised by using the 143 to 162 peptide fragment of rat α1-receptors as the antigen (see Experimental Procedures). α1 protein extracts from NG108 cells and rat hippocampus were prepared as described in Experimental Procedures. Thirty micrograms of P3 proteins from both NG108 cells and the rat hippocampus was used in this experiment. The immunoblots were performed with 1:2000 dilution of anti-α1-antibody. Hip, rat hippocampus. Numbers on the right side are molecular masses of protein standards, b, effect of (−)-PTZ. NG108 cells were treated with (−)-PTZ (100 nM) for 10 min. Thereafter the cytosol and P1, P2, and P3 subcellular fractions were prepared and analyzed with Western blotting. Control cells received no (−)-PTZ. P3 proteins from rat hippocampus also were included for comparison (hip). Note the increase of relative intensity of immunoreactive α1-receptors in the P1 and P2 fractions after (−)-PTZ treatment (bottom). c, effects of AS ODN and MS ODN treatments on the level of immunoreactive α1-receptors. See Experimental Procedures for details of ODN transfection. Thirty micrograms of P3 fraction from NG108 cells was electrophoresed on each lane. AS1, 48 h after transfection; AS2, 48 h after transfection; and MS, 48 h after transfection. AS ODN and MS ODN were dissolved in minimal amount of H2O before use. Control cells received only H2O.

Ca2+ signaling at the plasma membrane. To our knowledge, this is the first report suggesting that a Ca2+ signaling-affecting protein can be translocated from the ER to the plasma membrane. Thus, by extension, the endogenous α1-ligand such as PS may play an important role in the translocation of α1-receptors. Because (−)-PTZ is a relatively well known α1-ligand (Quirion et al., 1992; Hellewell et al., 1994), we examined in this study only the effect of (−)-PTZ on α1-receptor translocation. Effects of other α1-ligands such as PRE and PS will be examined in future experiments.

The effective concentration of PS used in this study (1 μM) is close to the Kd value of PS at the α-receptors (3 μM). This concentration of PS may raise a question whether the effect of PS seen in this study is physiologically relevant. The basal concentration of PS in rat brain hippocampus is typically ~12 ng/g (Vallee et al., 1997). However, in different regions of the brain, neurosteroid concentrations may vary according to environmental and behavioral circumstances, such as stress, sex recognition, and aggressiveness (Baulieu, 1998). It remains to be determined whether PS concentration can be raised, at least locally, in the brain to a low micromolar concentration that might affect Ca2+ signaling via α1-recep-
A progesterone-binding protein with certain similarity, but not totally the same as σ₁-receptors, has been identified in porcine liver and was suggested to be a member of the σ₁-receptor superfamily with a progesterone specificity (Falkenstein et al., 1998). However, the sequence of the protein does not resemble σ₁-receptors except that they both possess only one transmembrane region.

There are reports showing that σ₂-receptors also exist in NG108 cells (Georg and Friedl, 1991). Our results with the AS ODN directed against cloned σ₁-receptors suggest that σ₁-receptors modulate VDCC. Klette et al. (1997) reported that σ₁-receptors inhibited KCl-induced [Ca²⁺]i increases in primary cultured neurons. However, the effective concentrations of σ₁-ligands in that study are typically in the medium to high micromolar range and no agonist-antagonist relations were observed. It is not known whether σ₁-receptors mediate the effects described by Klette et al. (1997). It has been reported that the σ₁-receptor shares some pharmacological similarities with the phenylalkylamine Ca²⁺ antagonist emopamil-binding protein (Moebius et al., 1993). The sequences of these two proteins, however, are different.

Our present results showing that a 10-min preincubation of cells with σ₁-ligands was required to see an effect are in a way in resonance with previous reports demonstrating that at least a 10-min incubation of tissue with σ₁-ligands is required to see the resultant protein phosphorylation (Brent et al., 1997) or PI turnover (Bowen et al., 1988). Furthermore, it is interesting but also puzzling to note that in this study, the 10-min preincubation of cells with σ₁-ligands as a requirement to see an effect was observed both in the intracellular studies involving BDK and in the plasma membrane studies involving KCl-induced depolarization. Although the translocation of σ₁-receptors from the ER to the plasma membrane (Fig. 7) may explain the 10-min delay seen with the depolarization experiment, the translocation cannot explain the BDK results. The exact reason for the required 10-min incubation to detect these effects is thus unknown at present. The delayed time course of action may reflect an as-yet-to-be investigated biochemical nature of σ₁-receptors.

In conclusion, because σ₁-receptors are widely distributed in the nervous, endocrine, peripheral, and immune systems (Su, 1991; Hanner et al., 1996), our results demonstrating the modulatory action of σ₁-receptors on intracellular Ca²⁺ concentration suggest that σ₁-receptors may play a wide spectrum of physiological roles by acting as a unique, one-transmembrane protein affecting Ca²⁺ signaling in cells.

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