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Nonopioid Actions of the κ-Opioid Receptor Agonists, U 50488H and U 69593, on Electrophysiologic Properties of Hippocampal CA3 Neurons *in Vitro*[1]

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ABSTRACT

The actions of the nonpeptide κ-opioid receptor agonists, U 50488H (1–100 μM) and U 69593 (50–200 μM), on guinea pig hippocampal CA3 neurons were investigated *in vitro* by means of intra- and extracellular recording techniques. The compounds reduced the efficacy of synaptic transmission at the mossy fiber-CA3 synapse, and, simultaneously, enhanced the neuronal direct excitability. Intracellular recordings from CA3 neurons suggested two components underlying the drugs' excitatory actions: 1) Due to an apparent decrease in membrane leak conductance, the compounds enhanced the neuronal input resistance in a dose-dependent fashion. 2) The fast after-hyperpolarization following spontaneous or evoked action potentials was found to be substantially impaired in the presence of the drugs. In addition, extra- and intracellular recordings provided evidence that, by reducing the fast sodium conductance, both compounds exerted a local anesthetic action. The effects of U 50488H were antagonized neither by naloxone (2–50 μM) nor by the κ-opioid receptor antagonist, nor-binaltorphimine (10–20 μM), indicating that the drug-induced effects represent unspecific actions not linked to activation of opioid receptors.

Immunohistochemical studies have revealed the existence of distinct opioid peptide systems within the hippocampal formation: predominantly proenkephalin-derived peptides were found in the pathway from the entorhinal cortex to the dentate gyrus, and also in interneurons in both the CA1 and CA3 subfields, whereas prodynorphin-derived peptides were preferentially located in the dentate gyrus-CA3 mossy fiber pathway (see Henriksen *et al.*, 1988, for review). Regarding the electrophysiologic actions of opioid peptides in the hippocampus, μ- and δ-opioid receptor agonists have been found to excite hippocampal pyramidal cells due to a suppression of the activity of inhibitory interneurons (Zieglgänsberger *et al.*, 1979; Masukawa and Prince, 1982; Madison and Nicoll, 1988). In the CA3 region, dynorphin, which is an endogenous ligand at the κ-opioid receptor subtype (Chavkin *et al.*, 1982), but might also act on other opioid receptor subtypes (Chavkin *et al.*, 1985), has been reported to have both excitatory and inhibitory actions (Moises and Walker, 1985; Iwama *et al.*, 1986; see Chavkin *et al.*, 1988, for review). Since mainly the inhibitory effects of dynorphin were mimicked by κ-opioid receptor agonists, it has been suggested that inhibition by dynorphin is mediated *via* κ-opioid receptors, whereas the peptide's excitatory effects might be due to a disinhibition similar to that produced by enkephalins acting on μ- and δ-opioid receptors (Henriksen *et al.*, 1988). The present study was designed to disclose whether two widely used, stable nonpeptide ligands of the κ-opioid receptor, U 50488H (VonVoigtlander *et al.*, 1983) and U 69593 (Lahti *et al.*, 1985), would represent useful pharmacologic tools to identify directly electrophysiologic effects linked to activation of κ-opioid receptors in hippocampal area CA3 *in vitro*. Our data, however, provide evidence that the actions of both compounds, despite their high affinity to κ-binding sites in hippocampus, were apparently not mediated by κ-opioid receptors.

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Methods

Transverse slices (500 μm) were prepared from the hippocampus of ether-anesthetized guinea pigs (200–300 g) and kept submerged in ACSF containing (mM): NaCl, 118; KCl, 3; NaH_2PO_4, 1.25; $NaHCO_3$, 25; $MgCl_2$, 1.3; $CaCl_2$, 2; D-glucose, 10. The solution was gassed continuously with a mixture consisting of 95% O_2 and 5% CO_2 in order to obtain a pH value of 7.4 at a temperature of 30–31°C.

Electrical stimulation of the mossy fiber input to CA3 was performed using concentric steel electrodes. Dendritic field responses were recorded in stratum radiatum of area CA3 by means of glass microelectrodes filled with 3 M NaCl (resistance, 2–4 MΩ). Intracellular signals obtained from CA3 pyramidal neurons were recorded using glass microelectrodes filled with 3 M KCl (resistance, 40–80 MΩ). The recorded

ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization; AP, action potential; I/V, current-voltage; nor-BNI, nor-binaltorphimine; PSP, postsynaptic potential; RMP, resting membrane potential; R_N, input resistance; RR, rectification ratio; TTX, tetrodotoxin.

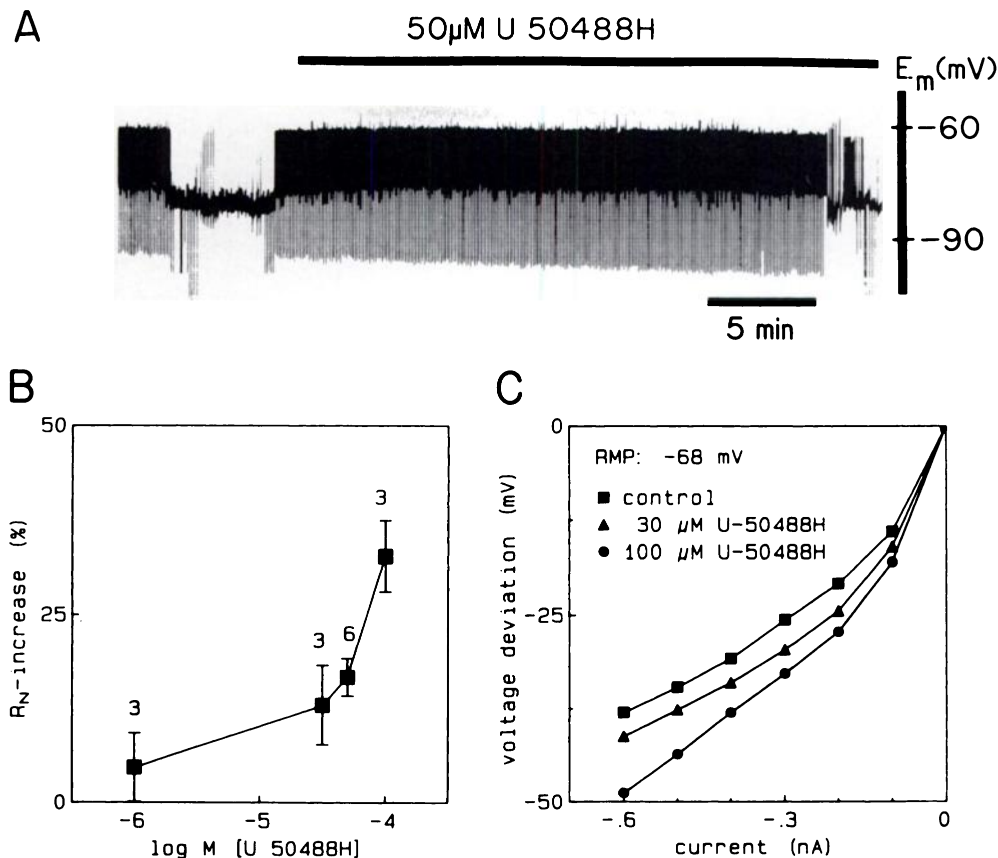

Fig. 1. Effects of U 50488H on RMP, R_N and I/V relationship. A: Continuous chart record of the membrane potential of a CA3 neuron. Downward deflections represent negative voltage deviations evoked by hyperpolarizing current injections, which were given to monitor R_N. Due to the slow time scale of the recording and the neuron's high frequent discharge of spontaneous APs, the corresponding upward deflections are condensed to a thick black bar. For measurements of the neuron's I/V relationship (data not shown), the membrane potential was hyperpolarized by negative DC-injection, until spontaneous activity ceased. Note the gradual increase in R_N following application of U 50488H, while RMP remained unchanged. U 50488H did not substantially affect the spontaneous discharge pattern of the neuron. B: Dose-response curve for the drug-induced increase in R_N. Bars represent S.D., numbers indicate number of experiments performed. C: Steady-state I/V relationship in the hyperpolarizing direction of a CA3 neuron (RMP: −68 mV; R_N: 104 MΩ) in the absence and presence of two concentrations of U 50488H. I/V curves were obtained by plotting the voltage deviation measured at the end of each pulse as a function of the amplitude of the corresponding current injection. The effects of U 50488H on R_N and the I/V relationship were not completely reversible even after prolonged superfusion with normal bathing solution (>2 hr).

signals were amplified by means of a switched current-clamp amplifier and continuously monitored on a chart recorder. The R_N was determined by injection of hyperpolarizing current pulses (0.2 nA, 500 msec). Neurons were selected on the basis of their RMP (>−60 mV), their R_N (>70 MΩ) and the spike overshoot (>15 mV). Recorded signals were digitized (sampling rate ≥5 KHz) and stored using a laboratory computer system.

TTX, 4-aminopyridine and naloxone were obtained from Sigma (Taufkirchen, Federal Republic of Germany), and nor-BNI was purchased from RBI (Natick, MA). All drugs were dissolved in ACSF. U 50488H and U 69593 were freshly dissolved in distilled water or in dimethyl sulfoxide, respectively, and added to ACSF to reach final concentrations between 1 and 100 μM (U50488H) or 50 to 200 μM (U 69593). With respect to U 69593, final solvent concentrations were ≤0.8%. At this concentration, dimethyl sulfoxide did not mimic the effects of U 69593 when applied in control experiments (n = 5). All substances were bath-applied by a superfusion system, the flow rate of which was set to 4 to 6 ml/min. Values are given as mean ± S.D. Statistical analysis was based on the paired Student's t test.

Results

Effects of U 50488H on RMP, R_N and the I/V relationship. When bath-applied in concentrations between 1 and 100 μM, U 50488H was found to concentration-dependently enhance R_N (control value: 90.2 ± 9.4 MΩ, n = 26). Within 5 to 8 min following application of U 50488H, the R_N increase began to develop, requiring about 30 min to reach steady state (fig. 1A). The dose-response curve for the U 50488H-induced R_N increase (fig. 1B) indicated that this effect appeared already at a concentration of 1 μM and attained statistical significance at concentrations equal to or above 30 μM. In contrast to its effect on R_N, U 50488H did not alter the neurons' RMP (control value: −66.8 ± 3.4 mV, n = 26) which remained unchanged within a range of ± 2 mV. Since it has been reported that U 50488H (100 μM) reduced anomalous rectification in hippocampal CA3 neurons (Iwama *et al.*, 1987), we examined whether the U 50488H-induced R_N increase might be due to an alteration in the properties of the inward (anomalous) rectifier. When determined under control conditions, steady-state I/V relationships of CA3 neurons typically showed inward rectification in the hyperpolarizing direction (fig. 1C, squares). In order to quantify the neurons' inward rectification, a RR was calculated from the resistances obtained by injecting small (0.2 nA) and strong (0.6 nA) hyperpolarizing current pulses, respectively. In drug-free ACSF, the RR was found to be 1.52 ± 0.13 (n = 3), indicating inward rectification. In the same neurons, U 50488H (50–100 μM) did not produce a significant change in the RR

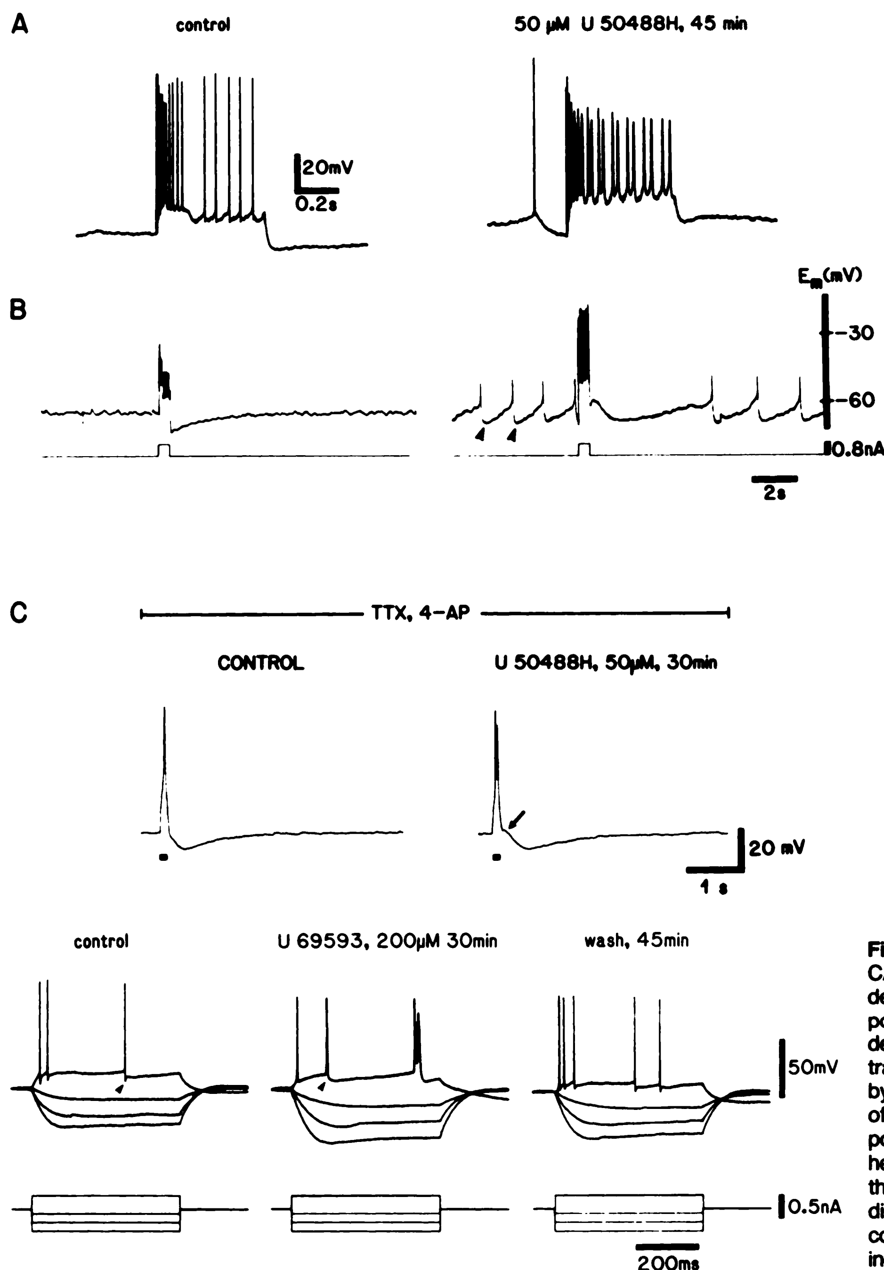

Fig. 2. Effects of U 50488H on the sequence of afterpotentials. A: Response of CA3 neuron to a depolarizing current pulse (0.8 nA, 500 msec) recorded under control conditions (left) and in the presence of 50 μM U 50488H (right). For details see text. B: Chart record of the depolarization-evoked discharges of A, shown on a slower time scale (top trace). The current trace below indicates time and amplitude of current injection. Note that due to the low-pass filtering of the chart recorder, fast signals were substantially attenuated. Under control conditions, the neuron's membrane potential was slightly below threshold for endogenous pacemaking activity. In the presence of U 50488H, however, the neuron displayed spontaneous rhythmic activity. This might be accounted for by the drug-induced increase in R_N, which in turn would amplify membrane conductances including those underlying pacemaking activity. As indicated by the arrowheads, the slowly decaying spike AHPs were apparently not abolished by U 50488H. C: Chart records of TTX-insensitive, putative calcium spikes evoked by short depolarizing current injections (1 nA, 50 msec). ■, Time of stimulation. In the presence of U 50488H, spike repolarization stopped above RMP and allowed the appearance of a depolarizing afterpotential (arrow), whereas the late AHP remained unchanged.

Fig. 3. Effects of U 69593 on intrinsic properties of a CA3 neuron. The neuron's intrinsic properties were determined by recording the deviation of the membrane potential (top trace, baseline: −68 mV) in response to de- and hyperpolarizing current injections (bottom trace). Under control conditions, spikes were followed by a fast and a slow AHP (arrowhead). In the presence of 200 μM U 69593, however, only the late AHP component was found to be resistant to the drug (arrowhead). The impaired spike repolarization also prompted the release of a second spike yielding a doublet-like discharge. While the early spike AHP completely recovered following drug-washout, the drug-induced R_N increase was only partially reversed.

(1.62 ± 0.15, n = 3), suggesting that the drug-induced shift of the I/V curves (fig. 1C, triangles and circles) could not be accounted for by a reduction in anomalous rectification.

Effects of U 50488H on afterpotentials. Besides its action on R_N and the I/V relationship, U 50488H was also found to affect the discharge behavior of CA3 neurons. Under control conditions (fig. 2A, left), a strong depolarizing current pulse (0.8 nA, 500 msec) initially elicited a rapidly adapting burst of APs followed by a regular discharge of single spikes. At the end of the pulse, the discharge was immediately followed by an AHP, which slowly decayed (fig. 2B, left). In the presence of 50 μM U 50488H, a high-frequency discharge of "doublets" was observed, indicating impaired spike repolarization (fig. 2A, right). When the current pulse was switched off, a sequence of an afterdepolarization followed by an afterhyperpolarization appeared (fig. 2B, right). As indicated by the slowly decaying AHPs, which followed spontaneously generated APs in the presence of U 50488H (fig. 2B, arrowheads), the drug's inhibitory action on AHPs was apparently limited to their fast components participating in spike repolarization, but did not extend to their slow components, which mediated the late AHP.

In order to avoid a possible contamination of AHPs by spontaneous or reverberating synaptic potentials, the action of U 50488H on AHPs was further investigated in TTX (0.6–1.2 μM)-treated slices. In the presence of 4-aminopyridine (0.1 μM), which selectively blocks a transient outward potassium current (Gustafsson *et al.,* 1982), short depolarizing current injections reliably evoked TTX-insensitive, putative calcium spikes, which were typically followed by a sequence of an early (fast) and late AHP (fig. 2C, left). Following application of 50 μM U 50488H, spike repolarization was again found to be impaired, prompting the discharge of a second spike. As indicated by the arrow in Fig. 2C, the suppression of the early AHP gave place to the appearance of an afterdepolarization, whereas the late AHP remained unchanged.

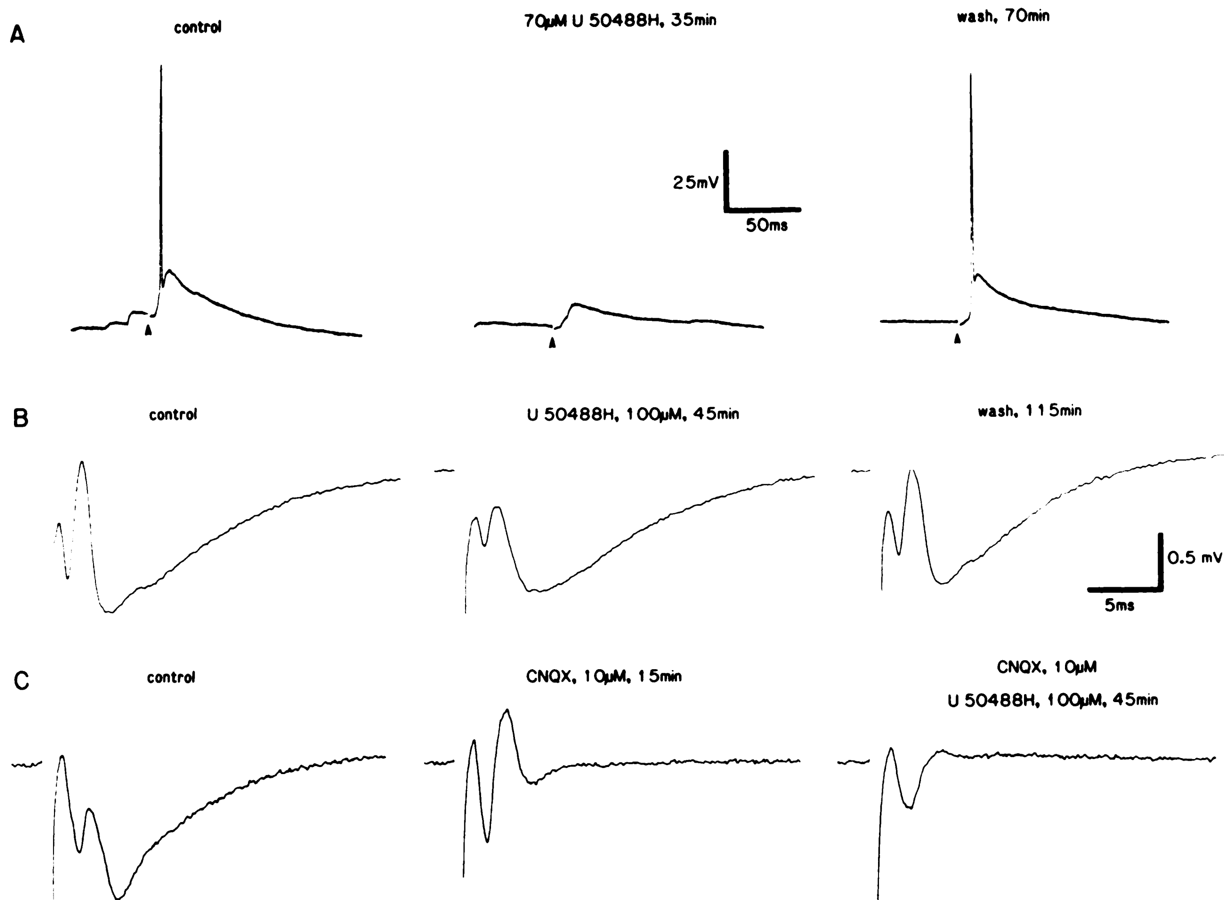

Fig. 4. Effects of U 50488H on PSPs. A: Intracellular recording from a CA3 neuron (RMP: −76 mV). PSPs were evoked by suprathreshold stimulus intensities, which were about 1.5 times above the stimulus strength required for eliciting a single spike (stimulus artifacts removed, arrowheads indicate time of stimulation). Due to the use of KCl-filled microelectrodes, the depolarizing envelope consisted of both an excitatoryPSP and an inhibitoryPSP followed by an hyperpolarizing late inhibitoryPSP of which only the first part is shown. U 50488H exerted an inhibitory action on PSPs, which, however, was observed only at higher concentrations. Note the reduction in spike amplitude in the sample recorded after drug wash-out. B: Field-PSPs elicited by electrical stimulation of the mossy fiber pathway were recorded in stratum radiatum of area CA3. For illustration, the upstroke of the stimulus artifact was removed. The first negative (downward) deflection following the downstroke of the artifact represents the afferent volley, the second, slowly decaying negative deflection caused by the field PSP. U 50488H simultaneously decreased the amplitude of field PSP and afferent volley. C: Same stimulus protocol as above. The field PSP was suppressed by CNQX (10 μM) leaving the afferent volley, which was substantially reduced by U 50488H.

In addition to its action on the fast AHP, U 50488H dose-dependently reduced the amplitude of sodium-dependent APs. When tested at a concentration of 50 μM, U 50488H significantly decreased the amplitude of individual spikes by 12.7 ± 3.3% ($n = 6$, $P < .0001$). This effect, which required about 40 to 50 min to develop, was not or was only partially reversible following prolonged superfusion with normal ACSF.

Effects of U 69593 on intrinsic properties of CA3 neurons. In one set of experiments ($n = 3$), we studied the actions of another representative of the class of stable nonpeptide κ-opioid receptor ligands, U 69593, using the same experimental protocols. When applied in concentrations between 50 and 200 μM, U 69593 dose-dependently increased R_N by 10 to 30% (fig. 3). At a concentration of 200 μM, the compound had additional effects on the neuronal discharge behavior evoked by depolarizing current injections. As illustrated in fig. 3 (middle panel), U 69593 reduced spike amplitude, abolished the early spike-AHP (arrowhead) and, thus, allowed the generation of doublet-like discharges. As for U 50488H, measurements of the I/V relationship in the absence and presence of U 69593 did not yield evidence for a reduction of inward rectification in the hyperpolarizing direction (data not shown). Compared with U 50488H, the actions of U 69593 required a moderately higher concentration range, but less time to develop (15–20 min of superfusion), and were more rapidly reversed in normal ACSF (30–40 min).

Effects of U 50488H and U 69593 on PSPs. A possible drug action on synaptic transmission was studied both intra- and extracellularly. In intracellular recordings, high concentrations of both U 50488H (≥50 μM) and U 69593 (200 μM, data not shown) consistently reduced PSP amplitude and increased threshold stimulus intensity in all neurons tested ($n = 8$, fig. 4A). To disclose whether the PSP reduction might be due to a local anesthetic action of the drugs, extracellular recordings of stimulus-evoked dendritic field responses were performed allowing simultaneous recording of afferent volley and field PSP. In these experiments, the drug-induced decrease in slope and amplitude of the field PSP was always accompanied by a decrease in amplitude and synchrony of the afferent volley, indicating a local anesthetic drug action (fig. 4B). Similar effects were obtained in the presence of 200 μM U 69593, which also produced a simultaneous decrease in the amplitude of the

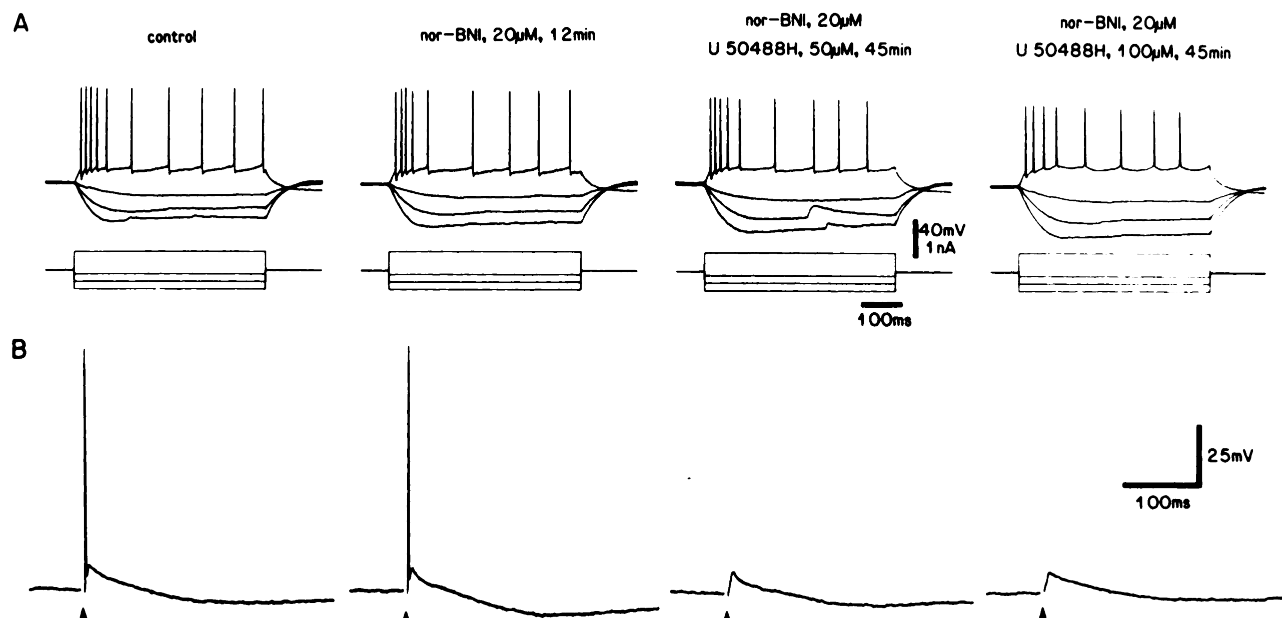

Fig. 5. nor-BNI insensitivity of the effects of U 50488H. A: Responses of the membrane potential of a CA3 neuron (top trace, baseline −69 mV) to de- and hyperpolarizing current injections (bottom trace) under control conditions, in the presence of the antagonist, and in two solutions containing both agonist and antagonist at different concentrations. For explanation see text. B: Intracellular recording of PSPs (membrane potential: −69 mV) evoked at suprathreshold stimulus intensity. Nor-BNI alone slightly enhanced PSPs. In the presence of nor-BNI, U 50488H equally reduced the amplitudes of the excitatoryPSP-inhibitoryPSP sequence as observed in antagonist-free solution (see fig. 4A).

field-PSP (mean reduction: 31.7 ± 13%, $n = 3$) and the afferent volley (data not shown).

Since a quantitative analysis of the afferent volley was somewhat hampered by its contamination with field PSPs, the volley was functionally isolated by blocking the PSP. For this purpose, slices were superfused with the glutamatergic, non-N-methyl-D-aspartate receptor antagonist, 6-cyano-7-nitroquinoxalin-2,3-dion (CNQX) (Andreasen *et al.*, 1989), which suppressed postsynaptic responses within 10 to 15 min following bath application (fig. 4C). Under these conditions, U 50488H (100 μM) again broadened the afferent volley and reduced its amplitude by 39.2 ± 8.4% ($n = 3$).

Effects of opioid receptor antagonists on the actions of U 50488H. In order to determine, whether the actions of U 50488H were due to κ-opioid receptor activation, we initially tried to block the drug action by the rather unselective opioid-receptor antagonist, naloxone (2–50 μM, $n = 3$). In these experiments, none of the effects of U 50488H were found to be antagonized by naloxone. Instead, naloxone enhanced rather than antagonized the effects of U 50488H on R_N, indicating intrinsic activity of the antagonist. Therefore, we used the novel and selective κ-opioid receptor antagonist, nor-BNI (Takemori *et al.*, 1988). When superfused under control conditions, nor-BNI (10–20 μM) enhanced R_N by about 5 to 10% and slightly increased the amplitude of PSPs in two of five neurons (see fig. 5, left), whereas these parameters remained unchanged in the other cells. Following addition to a nor-BNI (10–20 μM)-containing bathing solution, U 50488H (50–100 μM) was still able to produce its characteristic actions. As illustrated in figure 5 (right), nor-BNI blocked neither the U 50488H-induced R_N-increase, nor the suppression of spike-AHP, the reduction of spike amplitude or the inhibitory action on PSPs.

Discussion

In guinea pig hippocampal area CA3, the nonpeptide κ-opioid receptor ligands, U 50488H and U 69593, were found to have both inhibitory and excitatory actions within the same concentration range. Whereas the compounds reduced the efficacy of synaptic transmission at the mossy fiber-CA3 synapse, they simultaneously enhanced the neurons' direct excitability. With respect to the latter, intracellular recordings from CA3 neurons revealed several mechanisms that might underlie the drug-induced changes in the cells' intrinsic properties. Although RMP remained stable within a narrow voltage range (±2 mV), both ligands increased R_N in a concentration-dependent, voltage-insensitive manner, indicating reduction of a membrane leak conductance. Besides their action on R_N, the ligands substantially impaired spike repolarization. Whereas the former effects began to develop in the low micromolar range, spike repolarization was affected only at higher concentrations of U 50488H (≥50 μM) and U 69593 (200 μM), respectively (figs. 2 and 3). As an expression of impaired spike repolarization, the fast spike-AHP was found to be abolished. Instead, a depolarizing afterpotential appeared, which often gave rise to a second spike discharge (figs. 2 and 3). Our experiments do not allow to specify the conductance(s) causing the alterations in spike repolarization. A reduction of calcium currents, which is probably the major ionic mechanism of κ-opioid receptor agonists (North, 1986), is unlikely to account for this effect, since the late AHP, which is, like the fast AHP, mediated by a calcium-dependent K-conductance (Lancaster and Adams, 1986) remained unchanged in the presence of the drug (fig. 2).

In addition to their excitatory actions, both compounds decreased PSPs recorded intra- and extracellularly. Although we cannot exclude that the compounds might also affect presynaptic calcium currents, our results suggest that the drug-induced reduction in synaptic efficacy is at least partially mediated by local anesthesia. The evidence gathered for this conclusion is 2-fold: 1) The amplitude of TTX-sensitive, sodium-dependent APs was significantly attenuated in the presence of the drugs (figs. 2, 3 and 5). 2) Inhibition of field-PSPs

consistently coincided with a decrease in the amplitude of the afferent volley (fig. 4).

In view of the high affinity of U 50488H and U 69593 to the κ-opioid receptor in guinea pig brain (K_i values, 2.4 nM and 20.8 nM, respectively; Zukin *et al.*, 1988) and the high density of κ-opioid receptors in area CA3 (McLean *et al.*, 1987), it was quite surprising that electrophysiologic actions of the drugs were observed only at rather high concentrations. Thus, the question arose as to whether the ligands might have only a low intrinsic activity at κ-opioid receptors, or whether the drug effects might be related to mechanisms other than the activation of opioid receptors. Although the actions of U 50488H were apparently naloxone-insensitive, we could not exclude that the intrinsic activity of naloxone observed already in the low micromolar range might have masked a naloxone-U 50488H interaction at the κ-opioid receptor. Therefore, experiments were repeated using the novel κ-opioid receptor antagonist, nor-BNI (Takemori *et al.*, 1988). Again, none of the electrophysiologic effects of U 50488H were found to be antagonized (fig. 5). It should be noted that, at the concentration used (10–20 μM), nor-BNI acts no longer as a selective κ-antagonist so that μ- and δ-opioid receptors would be also saturated (Takemori *et al.*, 1988). We thus conclude that in the hippocampal slice, the electrophysiologic actions of U 50488H and, by analogy, U 69593, represented nonopioid actions not linked to activation of κ- or other opioid receptor subtypes.

Although apparently not suitable for studying κ-receptor-mediated neuromodulation in hippocampus *in vitro*, the compounds might be of value to identify putative nonopioid actions exerted by endogenous peptides. For example, Moises and Walker (1985), performing single-unit recordings on hippocampal pyramidal cells *in vivo*, reported that inhibition of cell firing by the endogenous κ-agonist, dynorphin, was not blocked by naloxone, but was mimicked by des-Tyr-dynorphine, a peptide fragment that is virtually devoid of any opiate-binding potential. Therefore, the authors raised the possibility that some of the biologically significant actions of dynorphin might be nonopiate in nature. It remains to be determined whether the complex and often opposing actions of dynorphin on central nervous system neurons (Sutor and Zieglgänsberger, 1984; Chavkin *et al.*, 1988; Henriksen *et al.*, 1988) might be partially due to one or several of the nonopioidergic mechanisms mediating the electrophysiologic actions of U 50488H and U 69593 on hippocampal CA3 neurons *in vitro*.

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