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Effects of DAGO on the rodent hippocampal evoked potentials using different perfusion solutions

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Abstract. Opioid receptor agonists exert excitatory effects in the hippocampus by inhibiting GABA release. We report that the μ-opioid agonist, DAGO, increases the amplitude of the population spikes (PS) measured in the *stratum pyramidale* of the CA1 cell layer in mouse and rat hippocampal slices perfused with an artificial cerebrospinal fluid (ACSF), but not when perfused in Krebs solution. The $GABA_A$ agonist, 3-APS, induces inhibitory responses when perfused in either ACSF or Krebs. Also, the field excitatory postsynaptic potentials (EPSP) measured on *stratum radiatum* do not differ when the slice is perfused with either ACSF or Krebs. The increase in the amplitude of the PS induced by DAGO is not obtained when perfused in a modified ACSF whose concentration of $MgSO_4$ was lowered to its concentration in the Krebs solution (from 2.4 mM to 1.2 mM). Thus, changes in the concentration of $MgSO_4$ seem to be responsible for the different responses induced by DAGO.

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INTRODUCTION

In the hippocampus, opioid receptors are located on the interneurons near the pyramidal cells in the *stratum pyramidale* (Mansour et al. 1994).Their stimulation by opiate agonists generally results in excitatory effects by inhibiting the release of GABA from the interneurons which, in turn, causes a decreased inhibition of pyramidal neurons, and, hence, an excitatory effect (Zieglgänsberger et al. 1979, Dunwiddie et al. 1980, Dingledine 1981, Valentino and Dingledine 1982, Bostock et al. 1984, Lupica and Dunwiddie 1991, Capogna et al. 1993). This excitatory effect has often been shown by measuring the modifications in the amplitude of the extracellular evoked potentials recorded at the pyramidal layer of the CA1 region in the hippocampal slice preparation (Zieglgänsberger et al. 1979, Dunwiddie et al. 1980, Dingledine 1981, Valentino and Dingledine 1982, Bostock et al. 1984, Lupica and Dunwiddie 1991). In this *in vitro* model, the stimulation of Schaffer collateral-commissural fibers of CA3 induces the release of glutamate (Fleck et al. 1993) which depolarizes pyramidal neurons in CA1, and, thus, the corresponding evoked potentials (population spikes) can be measured extracellularly.

In our laboratory, we have performed studies using the mouse hippocampal *in vitro* slice preparation, to analyze the modulation of the $GABA_A$ receptor by steroids (SanMartín et al. 1996). While testing the effects produced by the μ-opiate selective agonist, DAGO on this preparation with the same technique and procedure, we were unable to record an excitatory effect due to DAGO. Attempting to identify possible causes, we detected two differences in the literature that could possibly account for this lack of effect. First, the perfusion solutions used by the different authors in this preparation are not all exactly the same, and also differed slightly from ours. Second, slices from the rat brain are the most widely used, instead of mouse-derived ones. In order to test whether the excitatory effect induced by DAGO could only be obtained under specific experimental conditions mainly arising form the perfusion solutions employed, the experiments reported here were undertaken. Therefore, we studied the effect of DAGO when different perfusion solutions (Krebs or ACSF) or different animal species (rats or mice) were used. The effect of the $GABA_A$ agonist, 3-amino propane sulphonic acid (3-APS), was also tested. Finally, changes in the ionic composition of the concentrations of NaCl and $MgSO_4$ in the ACSF solution were assayed.

METHODS

Drugs

DAGO ([D-Ala^2,*N*-Me-Phe^4,Gly^5-ol] – enkephalin) and 3-amino propane sulphonic acid (3-APS), were used. Both came from Sigma and were dissolved in the perfusion solutions.

Animals

Forty male mice, Swiss CD1, 40-50 days old (weighing 28-32 g) and 15 male rats, Wistar, 50-60 days old (weighing 230-270 g), from the Animalario de la Universidad de Oviedo (Reg. 33044 13A), exposed to a light-dark cycle of 12h and with free access to water and food, were employed.

Preparation of the slices

Mice or rats, under light ether anesthesia, were killed by demedullation. The skull was opened and the hippocampus quickly removed. Hippocampal slices (400 μm thick) were cut with a vibratome (Campden Inst., Ltd), immediately placed in a Petri dish filled with Krebs or an artificial cerebrospinal fluid (ACSF) solution saturated with 95% O_2 5% CO_2 and kept at room temperature. After a 60 min period, the slices were placed on the net of the recording chamber (Scottish submersion chamber) where they were completely submerged in warmed (28°C) and oxygenated (95% O_2 5% CO_2) perfusion solution (either Krebs or ACSF). The slices were weighed down with small pieces of platinum wire. The volume of the recording chamber was 1 ml and the perfusion solution (previously warmed by a heating jacket to 28°C) entered the recording chamber through the bottom and superfused the tissue at 1–1.5 ml/min. The Krebs solution had the following composition (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.5; $KH_2PO_4.7H_2O$, 1.19; $NaHCO_3$, 25; $MgSO_4$, 1.2 and glucose, 11; and the ACSF used (Lupica and Dunwiddie 1991) was composed as follows (mM): NaCl, 124; KCl, 4.9; $CaCl_2$, 2.5; $KH_2PO_4.7H_2O$, 1.2; $NaHCO_3$, 25.6; $MgSO_4$, 2.4 and glucose, 10. In additional experiments the composition of the ACSF was modified by decreasing the concentration of NaCl [NaCl] or $MgSO_4$ [$MgSO_4$] to reach the concentrations present in Krebs. Thus, a low[NaCl]-ACSF (NaCl, 118mM), and a low[$MgSO_4$]-ACSF ($MgSO_4$ 1.2 mM) were used.

Electrophysiological recordings

Electrophysiological recordings were made extracellularly in the CA1 cell layer as previously reported (SanMartín et al. 1996, SanMartín et al. 1999). The recording electrodes were borosilicate glass micropipettes (Clark Electromedical) filled with 3M NaCl, having a tip of 5–10 μm diameter yielding 2-5 MΩ of impedance. Inside the micropipettes an Ag/AgCl wire was connected by a 10 cm cable to the probe of a directly coupled (D.C. coupling) preamplifier (Cibertec AE-2). For electrical stimulation, a bipolar, twisted teflon insulated wire electrode (12.5 μm tip diameter) was used. Both electrodes were placed under visual control with the aid of micromanipulators (David Kopf Instruments) and a microscope (x 40, Wild Heerbrugg), the bottom of the bath being illuminated with an optic fiber illuminator (Intralux, Volpoi). The recording electrode was situated either at the pyramidal cell layer (*stratum pyramidale*) or at the *stratum radiatum* of the CA1 region to record population spikes and field excitatory postsynaptic potentials (EPSP), respectively. The stimulation electrode was placed at the *stratum radiatum* of CA3 to stimulate the Schaffer collateral-commissural fibre pathway. The stimuli were square-wave pulses of 200 μs duration, applied at a frequency of 0.1 Hz and at 15-25 V stimulus strength generated by a pulse programmer (Digitimer D 4030) and a stimulator (Digitimer 3072 Stimulator). Once amplified, the electrical potentials were monitored on a digital oscilloscope (Philips PM 3302) filtering those obtained at frequencies higher than 1 kHz. Finally, the recordings were saved for further analysis using the Powerlab MacLab/2e computer programme (ADInstruments). In those recordings made in the *stratum pyramidale*, the peak-to-peak size (mV) of the population spike was measured and when the recording corresponded to the *stratum radiatum*, the slope (mV/ms) of the field EPSPs was measured.

To begin the search for the typical recording, the stimulation frequency was set at 1 Hz, and once obtained, the voltage was increased in order to determine the supramaximal voltage. Then, the frequency was fixed at 0.1 Hz and a 30 min period was allowed in order to attain a stable recording. Afterwards, since we aimed to detect increases in the amplitude of the spikes, the voltage was lowered so that the amplitude of the population spikes decreased about 25%.This voltage was fixed to remain constant throughout the experiment. Finally, a further 30 min period was allowed before starting the drug session.

Experimental procedure

The drugs were added directly to the perfusion solution. To test the effects of the increasing concentrations of the drugs, the slices (either from mouse or rat brains were perfused with each concentration during 5 min (DAGO) or 3 min (3-APS), and the perfusion of each concentration was followed by a washout period of 15 or 10 min, respectively, to enable recovery. The recording of the EPSP in the *stratum radiatum* was made in slices from mice brains that had been initially perfused with ACSF for 15 min and immediately after with Krebs for a further 15 min. When the effect on the amplitude of the population spikes by the different perfusion solutions was tested in the presence of a single concentration of DAGO, the mouse hippocampal slices were sequentially perfused for 15 min with each solution without an intermediate washout period. In this set of experiments, DAGO was always initially perfused dissolved in ACSF and then, in the different experiments, in (1) Krebs, (2) low[NaCl]-ACSF and (3) low[$MgSO_4$]-ACSF.

In all cases, the effects of the drugs on the population spikes were measured at the end of each perfusing period and expressed as percentages of the baseline (predrug population spike) obtained immediately before perfusing in the presence of the drug. Only those slices in which the recovery by washout was at least 80% of the predrug (basal) response were included in the study. Each slice was used only once and the experiments were repeated at least 5 times in slices from different brains.

Data analysis

Data are expressed as means ± SEM. A two-way analysis of variance was undertaken to show the possible influence of the perfusion solution (ACSF or Krebs) on the effects induced by several doses of DAGO or 3-APS. Afterwards, the effect produced by the different concentrations of DAGO or 3APS in each perfusion solution was further compared with their untreated controls by a one-way analysis of variance followed by a Dunnett's *t* test to detect significant differences. To compare the EPSP initially measured in ACSF and then in Krebs, a Student's *t* test for paired data was used. The effect produced by a single concentration of DAGO in the presence of different perfusion

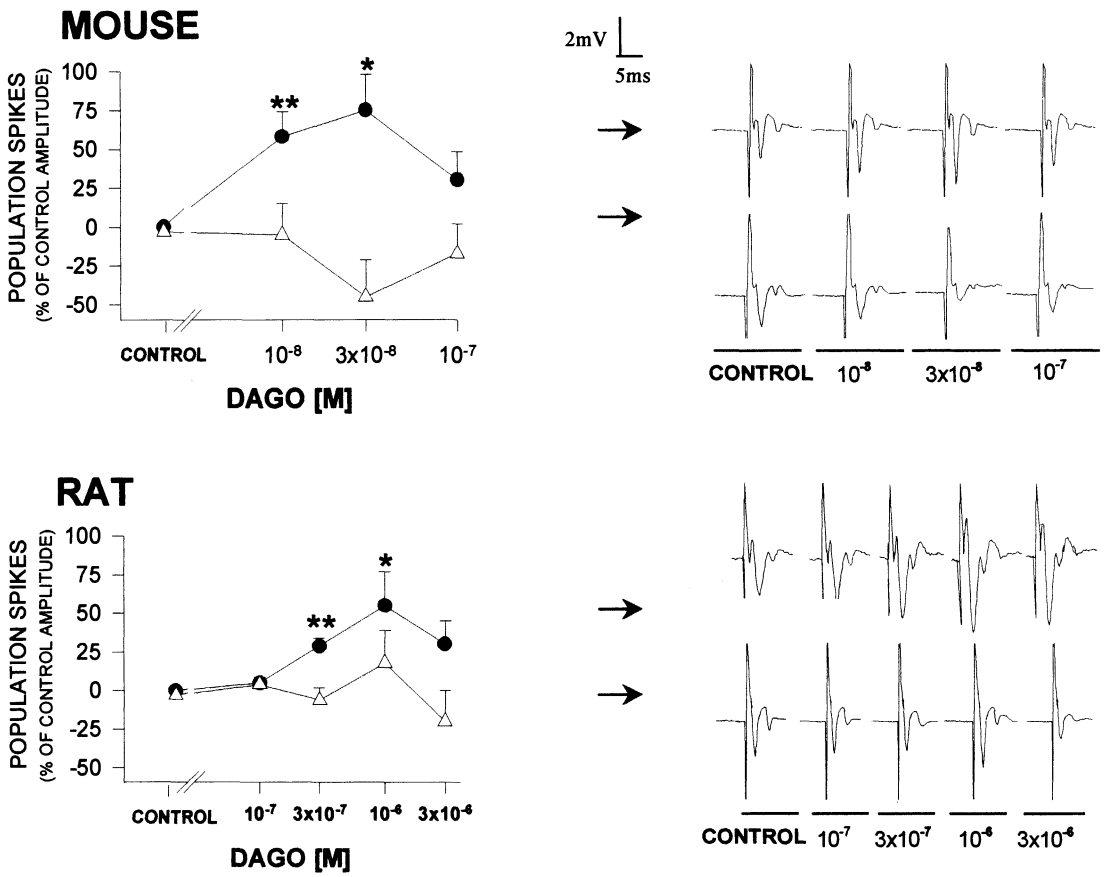

Fig. 1. Effects induced by the perfusion of DAGO on the amplitude of the population spikes measured in the *stratum pyramidale* of hippocampal slices from mice and rats. On the left hand side, concentration-effect curves in the presence of DAGO perfused in ACSF (closed symbols) and Krebs (open symbols). Each point represents the mean of the percentage of increase in the amplitude of the population spikes and their corresponding SEM ($n = 5$) . Comparisons are made with pre-drug administration, * $P<0.05$, ** $P<0.01$, Dunnett's t test. On the right hand side, examples of the amplitude of the population spikes produced during one particular experiment in each case (in the upper part, DAGO perfused in ACSF and in the lower part of each figure half, DAGO perfused in Krebs.

solutions was compared with the controls (in the absence of DAGO) by using a Student's t test for unpaired data. In all cases statistical significances were set at $P<0.05$.

RESULTS

The effects induced by DAGO (10^{-8} -10^{-7} M) on the amplitude of the population spikes measured in the *stratum pyramidale* of hippocampal slices from mouse brains were significantly different depending on the perfusion solution employed (ACSF or Krebs) as shown by the two-way analysis of variance ($F_{1,40}$ = 17.13, $P<0.01$). These concentrations of DAGO induced an increase in the amplitude of the population spikes when ACSF was the perfusion solution ($F_{3,23}$ = 5.58, $P<0.01$), but not when DAGO was dissolved in Krebs ($F_{3,23}$ = 0.14, N.S.) (Fig. 1A). In rat hippocampal slices, the effect induced by DAGO was also affected by the perfusion solution used (ACSF or Krebs in a significant way ($F_{1,48} = 7.56$, $P<0.05$) and a significant interaction between the treatment with DAGO and the perfusion solution was obtained ($F_{3,48}$ = 4.51, $P<0.05$). The perfusion with DAGO (10^{-7} - 10^{-6} M) dissolved in ACSF ($F_{3,34} = 12.1$, $P<0.01$), but not the perfusion of DAGO dissolved in Krebs ($F_{3,27} = 0.82$, NS), produced an increase in the amplitude of the population spikes measured in rat hippocampal slices (Fig. 1B).

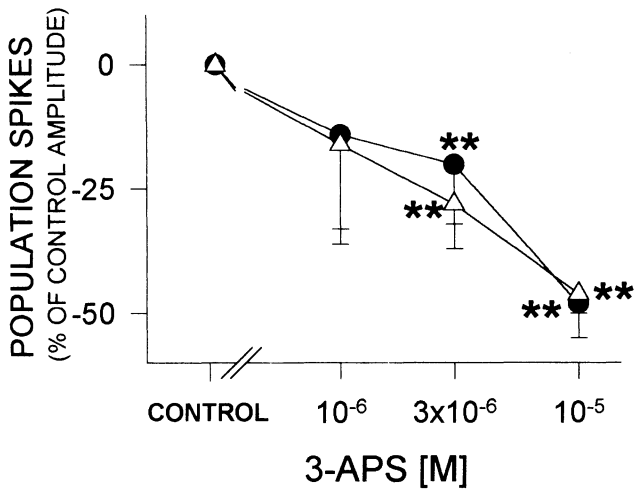

Fig. 2. Concentration-effect curves in the presence of 3-APS perfused in ACSF (closed symbols) and Krebs (open symbols) in mouse hippocampal slices. Each point represents the mean of the percentage of inhibition in the amplitude of the population spikes and their corresponding SEM (n = 5 data). Comparisons are made with pre-drug administration, ** P<0.01, Dunnett's t test.

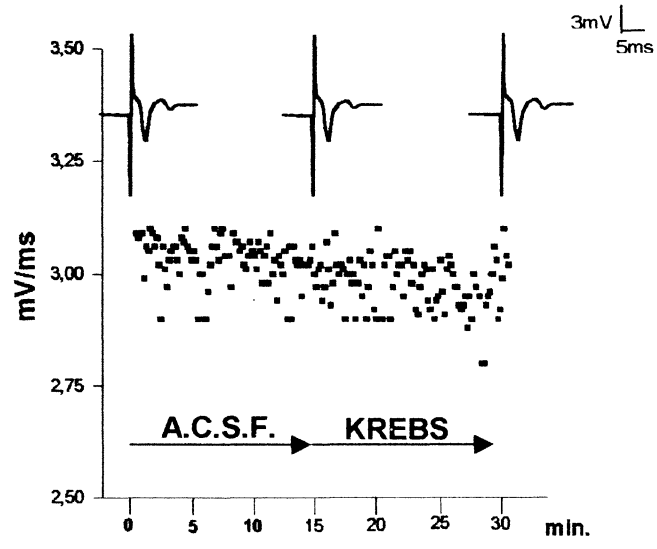

Fig. 3. Time course of the magnitude of the field EPSP (excitatory postsynaptic potentials) recordings obtained in the *stratum radiatum* of the CA1 region from one particular experiment in a mouse hippocampal slice initially perfused with ACSF and afterwards with Krebs. The lower part depicts the values of the magnitude of the EPSP throughout the experiment. In the upper part, three EPSP recordings corresponding to this experiment are shown.

To study whether the different perfusion solutions could modify the GABA-mediated transmission, we tested the effect of the $GABA_A$ agonist, 3-APS. The effect induced by this agonist showed no significant differences ($F_{1,40}$ = 0.026, NS) depending on whether the perfusion solution used to dissolve the 3-APS was either ACSF or Krebs. Increasing concentrations of 3-APS (10^{-6} - 10^{-5} M), brought about a significant decrease in the amplitude of the population spikes both in ACSF ($F_{3,23}$ = 22.9, P<0.01) and in Krebs solution ($F_{3,23}$ = 16.87, P<0.01) (Fig. 2).

We have also analyzed the possibility that, following the use of different perfusion solutions, modifications in the excitatory input could occur. For this purpose, we measured the field excitatory postsynaptic potential (EPSP) in the *stratum radiatum* of slices perfused initially with ACSF and immediately after with Krebs. The EPSP values were similar when the perfusion solution was changed in the same slice from ACSF (3.1 ± 0.73 mV/ms to Krebs (3.26 ± 0.72 mV/ms and no significant differences were obtained (t_4 = 0.83, NS) (Fig. 3).

When the mouse hippocampal slice was initially perfused with a single concentration of DAGO (3 x 10^{-6} M) dissolved in ACSF, an increase in the amplitude of the population spikes was recorded (t_{12} = 2.863, P<0.05). This increase was no longer measured in the same slice when immediately after it was perfused with the same concentration of DAGO dissolved in Krebs (t_{12} = 0.276, NS). Since the greatest difference between the composition of the ACSF and Krebs solutions lies in their concentration of NaCl and of $MgSO_4$, we studied whether these values could be critical to obtain excitatory effects by DAGO. Both [NaCl] and [$MgSO_4$] are higher in ACSF than in Krebs. Thus, experiments were undertaken testing initially the effect of a single concentration of DAGO perfused in ACSF and then, changing the perfusion solution (either low[NaCl]-ACSF or low[$MgSO_4$]-ACSF) in the same slice where the effect of DAGO was again measured (Fig. 4). The significant increase (t_{10} = 5.61, P<0.01) in the amplitude of the population spikes in slices perfused with DAGO dissolved in ACSF persisted when the perfusion solution was changed to the low[NaCl]-ACSF solution (t_8 = 5.14, P<0.01). On the contrary, the increase in the amplitude of the population spikes induced by DAGO initially perfused in ACSF (t_8 = 2.33, P<0.05) was no longer obtained when the perfusion solution was changed to low[$MgSO_4$]-ACSF solution (t_8 = 1.5, NS).

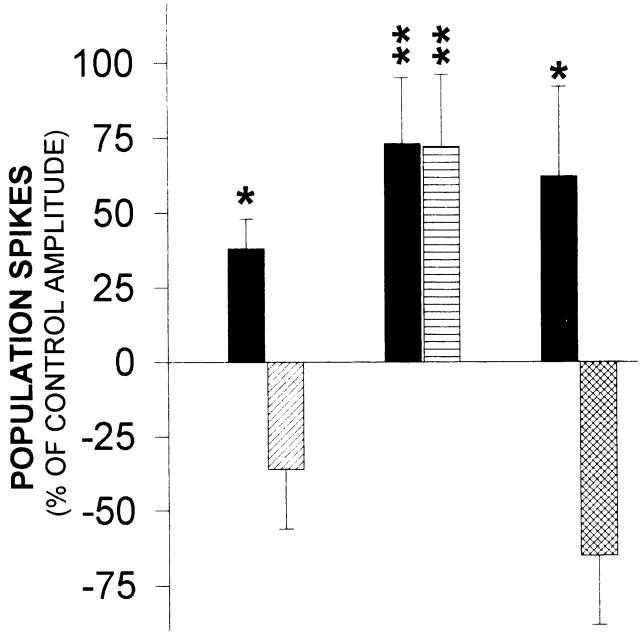

Fig. 4. Variations in the amplitude of the population spikes obtained in three independent experiments in the *stratum pyramidale* of the CA1 region of mice hippocampal slices. In all cases, the slices were continuously perfused with DAGO ($3x10^{-6}$ M), initially dissolved in ACSF (filled bars) and immediately afterwards in another solution: Krebs (obliquely hatched bars), low[NaCl]-ACSF (horizontally hatched) and low[MgSO4]-ACSF (chequered). The means of the percentage of increase in amplitude and their corresponding SEM ($n = 5$) are shown. Comparisons are made to pre-drug responses, $*P<0.05$, $**$ $P<0.01$, Student's t test for unpaired data.

DISCUSSION

Our results reveal that the selective μ opiate agonist, DAGO, produces excitatory effects in the mouse hippocampal slice preparation when an artificial cerebrospinal fluid (ACSF) is used as the perfusion solution. However, this excitatory effect was not observed when the perfusion was made in Krebs solution. Since the majority of studies with this preparation are performed in slices from rat brains we also tested the effects induced by DAGO in rat hippocampal slices. The different responses induced by the μ opiate agonist in each perfusion solution seem to be unrelated to the animal species because when rat hippocampal slices were employed, DAGO, once again, induced excitatory effects when perfused in ACSF but not in Krebs solution.

Afterwards, since the opiate agonist effects in the hippocampal slices are GABA-mediated, we analyzed whether the difference could be caused by a different GABA-induced response in the perfusion solutions used. This possibility was also ruled out because an inhibitory concentration-dependent effect was obtained in the presence of the $GABA_A$ agonist, 3-APS, perfused either in Krebs or in ACSF solution.

The stimulation of Schaffer collateral-commissural fibres of CA3 induces the release of glutamate (Fleck et al. 1993) which depolarizes pyramidal neurons in CA1. Thus, we studied whether differences in the synaptic input could occur following changes of the perfusion solutions that could be responsible for the different effects induced by DAGO in both solutions. However, the depolarization slope measured in the *stratum radiatum* remained unaltered when the perfusion solution was changed from ACSF to Krebs. Thus, an increased synaptic input did not seem to be responsible for the different effects of DAGO in either perfusion solutions.

Finally, we tested whether the different effect induced by DAGO on the mouse hippocampal slices perfused with Krebs or with ACSF could be explained by the different ionic compositions of the solutions. The main difference between the Krebs and the ACSF solution used lies in their concentration of NaCl ([NaCl]) and $MgSO_4$ ([$MgSO_4$]). Both concentrations are greater in ACSF than in Krebs ([NaCl]: 124 mM in ACSF and 118 mM in Krebs; [$MgSO_4$]: 2.4 mM in ACSF and 1.2 mM in Krebs). The increase in the amplitude of the population spikes obtained when a particular slice was perfused with DAGO dissolved in ACSF remained unmodified when a low[NaCl]-ACSF (in which the concentration of NaCl is the same as in Krebs) was used but returned to control (predrug levels) when the perfusion solution was changed to a low[$MgSO_4$]-ACSF (in which the concentration of $MgSO_4$ is the same as in Krebs). This result suggests that the concentration of $MgSO_4$ in the perfusion solution may be crucial to obtain increases in the amplitude of the population spikes in the presence of DAGO. Previous reports dealing with the effects produced by opiate agonists in this same preparation in rats show that the excitatory effects induced by them are generally obtained by using perfusion solutions in which the concentration of magnesium is usually above 1.5 mM, slightly higher than the one we used (1.2 mM) in the Krebs solution (Zieglgänsberger et al. 1979, Dunwiddie et al. 1980, Dingledine 1981, Valentino and Dingledine 1982, Bostock et al. 1984, Lupica and Dunwiddie 1991). Thus, it seems that small changes in the [$MgSO_4$], close to the physiological range of [Mg^{2+}]

in cerebrospinal fluid (1-2 mM), could determine the induction or not of excitatory effects by the administration of DAGO.

From these data it is tempting to consider what particular step in the action of DAGO (its affinity, its transduction processes or even, independently, a direct decrease in GABA release) could be affected by the lower $MgSO_4$ concentration. Some reports do not detect differences in the affinity of [^{3}H]DAGO to rat or guinea-pig brain membranes in the presence of various concentrations of Mg^{2+} ranging from 1 to 10 mM (Tejwani and Hanissian 1990, Rodríguez et al. 1992). Regarding transduction processes, it could be considered that a lowering of extracellular Mg^{2+} could affect the levels of intracellular Ca^{2+}. In isolated hippocampal CA1 pyramidal cells, only when the concentration of Mg^{2+} is lower than 0.3 mM (but not at concentrations of 1.2 mM or 4.8 mM, similar to those used here) is the intracellular Ca^{2+} concentration increased (Zhang et al. 1996). Finally, regarding the release of GABA, Fueta et al. (1995) reported a decrease in the occurrence and amplitude of GABA-mediated synchronous potentials in slices from rat hippocampi when extracellular [Mg^{2+}] was decreased from 2 mM to 1 mM. Since the increase of DAGO-induced excitability in this preparation is a consequence of the inhibition of GABA release, a probable lower release of GABA under low [Mg^{2+}] conditions (Krebs) could explain the lack of effect induced by DAGO in this perfusion solution.

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