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ORIGINAL INVESTIGATION

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Pharmacological demonstration of the differential involvement of protein kinase C isoforms in short- and long-term memory formation and retrieval of one-trial avoidance in rats

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Abstract *Rationale:* The hippocampal protein kinase C (PKC) family is involved in the early events of consolidation of long-term potentiation (LTP) and long-term memory (LTM). Results so far are indecisive about which PKC isoform is involved and as to whether any of them plays a role in short-term memory (STM) processes, which have recently been shown to be separate from those of LTM in the hippocampus-dependent one-trial step-down inhibitory avoidance task. *Objectives:* To measure the effect of two PKC inhibitors, one (Gö 6976) selective to the calcium-dependent isoforms α and βI, and the other (Gö 7874) unspecific as to PKC isoforms on the formation and retrieval of STM and LTM of one-trial inhibitory avoidance. *Methods:* Rats bilaterally implanted with cannulae in the CA1 region of the dorsal hippocampus were trained in one-trial step-down inhibitory avoidance. The effect of these two drugs on STM and LTM formation was investigated as follows. Animals were infused 10 min before or 50, 110, or 170 min after inhibitory avoidance training with a vehicle (2% dimethylsulfoxide in saline), or with Gö 6976 (0.92 nM or 4.6 nM) or Gö 7874 (1.96 nM or 8 nM) dissolved in the vehicle. Infusion volume was 0.5 µl in all cases. Animals were tested 1.5 h and 3 h after training for STM and at 24 h for LTM. In order to study the effects of these compounds on retrieval, they were infused into the hippocampus 10 min prior to STM testing at 3 h (see above) or 10 min before LTM testing at 24 h. In addition, the effect of Gö 6976 and Gö 7874 was studied on general activity measured in an open field, and on performance in an elevated plus maze. *Results:* STM was suppressed by 4.6 nM Gö 6976 given 10 min before or 50 min after training. LTM was cancelled by the higher dose of the two compounds given 10 min before, or 50 min or 110 min after training. None of the two compounds infused 170 min post-training affected the retrieval of STM measured 10 min later. However, both compounds given 10 min before testing inhibited the retrieval of LTM measured at 24 h. This effect cannot be attributed to influences on locomotor activity or anxiety levels, since the drugs had no effect on performance in the open field but were mildly "anxiogenic" (pro-conflict) and reduced the number of entries into open and closed arms and rearings. *Conclusions:* LTM consolidation requires in part α- and/or βI-PKC and in part other PKC isoforms. STM formation requires instead only α and/or βI-PKC and during a more limited period of time. In addition, PKC appears not to be necessary for the retrieval of STM, but is crucial for the retrieval of LTM. These findings further point to a biochemical separation of STM and LTM, as ascertained in numerous previous studies.

Keywords Protein kinase C · α/βI Protein kinase C · Short-term memory · Long-term memory

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Introduction

Protein kinase C (PKC) comprises a family of isoenzymes activated by synaptic agonist-induced rises in intracellular calcium and lipid mediators and has been extensively implicated in pre- and postsynaptic events related to neuronal plasticity (Huang et al. 1992; Colley and Routtenberg 1993; Mellor and Parker 1998; Micheau and Riedel 1999). Hippocampal PKC is crucial to the induction and early maintenance of long-term potentiation (LTP) (Abeliovich et al. 1993a; Colley and Routtenberg 1993) and the consolidation of long-term memory (LTM) of one-trial avoidance in rats (Jerusalinsky et al. 1994; Cammarota et al. 1997) and chicks (Burchuladze et al. 1990) and many other tasks in different species (Takashima et al. 1991; Mathis et al. 1992; Abeliovich et al. 1993b; Colombo

et al. 1997; Muzzio et al. 1997; Douma et al. 1998; Grunbaum and Muller 1998).

Currently, 12 isoforms of PKC have been characterized and classified according to their sensitivity to activators (Tanaka and Nihizuka 1994; Mellor and Parker 1998). The calcium-dependent conventional PKCs include the α, βI, βII and γ isoforms, activated by calcium, diacylglycerol, and other lipid mediators. Newly identified isoforms (nPKCs) δ, ε, η, and θ do not require calcium for their activation, and the atypical ζ and λ isoforms (aPKCs) are characterized by their insensitivity to classical PKC activators such as diacyglycerol and phorbol ester. The variety of isoenzyme activator mechanisms is accompanied by a selective distribution among brain areas and subcellular compartments, suggesting substrate specificity and differential physiological roles (Saito et al. 1993, 1994; Van der Zee et al. 1995; Mellor and Parker 1998).

Several studies indicate that hippocampal PKC is crucial to the formation of LTM of one-trial inhibitory avoidance. Bilateral inhibition of hippocampal PKC by staurosporin or CGP 41231 causes retrograde amnesia when infused into CA1 in the first 1–2 h after training (Jerusalinsky et al. 1994). PKC activation by intrahippocampal infusion of phorbol ester enhances memory of this task (Yang and Lee 1993). Training in this task triggers a learning-specific increase in membrane-bound PKC in CA1 and other hippocampal subregions (Bernabeu et al. 1995). Both membrane-bound total PKC activity and phosphorylation of its presynaptic substrate B50/GAP43 increase after one-trial avoidance with a peak 30 min post-training (Cammarota et al. 1997). A recent study indicates that this increase of PKC activity is mainly due to that of the βI isoform (Paratcha et al. 2000). In inhibitory avoidance in chicks, α and β isoforms of PKC also increase post-training (Bourtchuladze et al. 1995). In contrast, after food-rewarded spatial learning, the γ isoform is translocated to membranes (Douma et al. 1998), transgenic mice lacking γ-PKC are deficient in spatial tasks (Abeliovich et al. 1993b), and spatial learning induces a specific increase of hippocampal γ-PKC in proportion to the degree of learning (Van der Zee et al. 1997). It is possible that γ-PKC is more relevant to spatial than other associative forms of learning. However, both βII- and γ-PKC isoforms have been reported to increase following spatial learning in rats, and also in proportion to the degree of learning (Colombo et al. 1997).

Recent studies have shown that, at least for the one-trail step-down inhibitory avoidance task, a short-term memory (STM) system, separate from and parallel to LTM formation, operates in the hippocampus and in cortical areas connected with it (Izquierdo et al. 1999). This task has been exhaustively shown to depend on the hippocampus and on its afferent and efferent connections mediated by and involving the entorhinal cortex (Izquierdo and Medina 1997). Some mechanisms underlying STM overlap with those of LTM consolidation, but several others are different (Izquierdo et al. 1998a, 1998b, 1998c, 1999; Vianna et al. 1999). In all, sixteen different pharmacological treatments given into hippocampus, entorhinal, and parietal cortex selectively block the STM of one-trial avoidance while leaving LTM intact, which shows that STM is not a stage toward LTM but a separate system (Izquierdo et al. 1999). The analysis of the biochemistry of STM is complicated by its simultaneity with LTM consolidation (Izquierdo et al. 1998a, 1999). Recent experiments demonstrated the differential involvement of protein kinase A (PKA) (Vianna et al. 1999) and the mitogen-activated protein kinase (MAPK) pathway (Walz et al. 2000) on the two memory types: specific inhibitors of these enzymes given at different times after training affect STM and LTM separately.

The present study was designed to evaluate the contribution of PKC (Paratcha et al. 2000) to STM and LTM processing both during their formation and at the time of retrieval. Two distinct PKC inhibitors were chosen: one which selectively inhibits α- and βI-PKC (Gö 6976), and a generic PKC inhibitor (Gö 7874). Two different concentration levels of each drug were used: one close to their IC_{50}, and another one five times lower. The use of doses beyond that range is not recommendable. At higher concentrations, Gö 6976 affects other PKC isoenzymes, and Gö 7874 affects myosin kinase and PKA (Paratcha et al. 2000).

Materials and methods

Subjects

Two-hundred and seventy-one male Wistar rats from our own breeding colony were used (age 2.5–3 months; weight 215–300 g). They were bilaterally implanted under deep sodium thiopental anesthesia with 27-gauge guide cannulae aimed 1.0 mm above the pyramidal cell layer of the CA1 region of the dorsal hippocampus at coordinates A 4.3 mm, L 4.0 mm, and V 3.4, according to the atlas by Paxinos and Watson (1986). These parameters were the same as used in numerous previous experiments in which we established the deleterious effect on inhibitory avoidance of the reversible inactivation of various hippocampal receptors and signaling pathways (Izquierdo and Medina 1997).

Inhibitory avoidance training and infusion procedures

Once recovered from surgery, the rats were trained in one-trial step-down avoidance as described elsewhere (Jerusalinsky et al. 1994; Izquierdo et al. 1998a, 1998b, 1998c; Vianna et al. 1999). Briefly, animals were placed on a 2.5-cm high, 7-cm wide platform at the left of a 50-cm wide, 25-cm deep, 25-cm high inhibitory avoidance box, whose floor was a grid of 1-mm caliber bronze bars spaced 1 mm apart. In the training session, immediately after stepping down, placing the four paws on the grid, the rats received a 0.4-mA, 2-s scrambled foot shock.

Two experiments were carried out. Experiment 1 investigated the effect of the two PKC inhibitors on STM and LTM formation and on the retrieval of STM. Experiment 2 investigated the effect of the drugs on the retrieval of LTM, on open-field behavior, and on performance in an elevated plus maze (Pellow et al. 1985).

In experiment 1, the animals received bilateral 0.5-µl infusions of the vehicle (2% dimethylsulfoxide in saline), or of Gö 6976 (0.92 nM and 4.6 nM) or Gö 7474 (1.92 nM or 8 nM) dissolved in

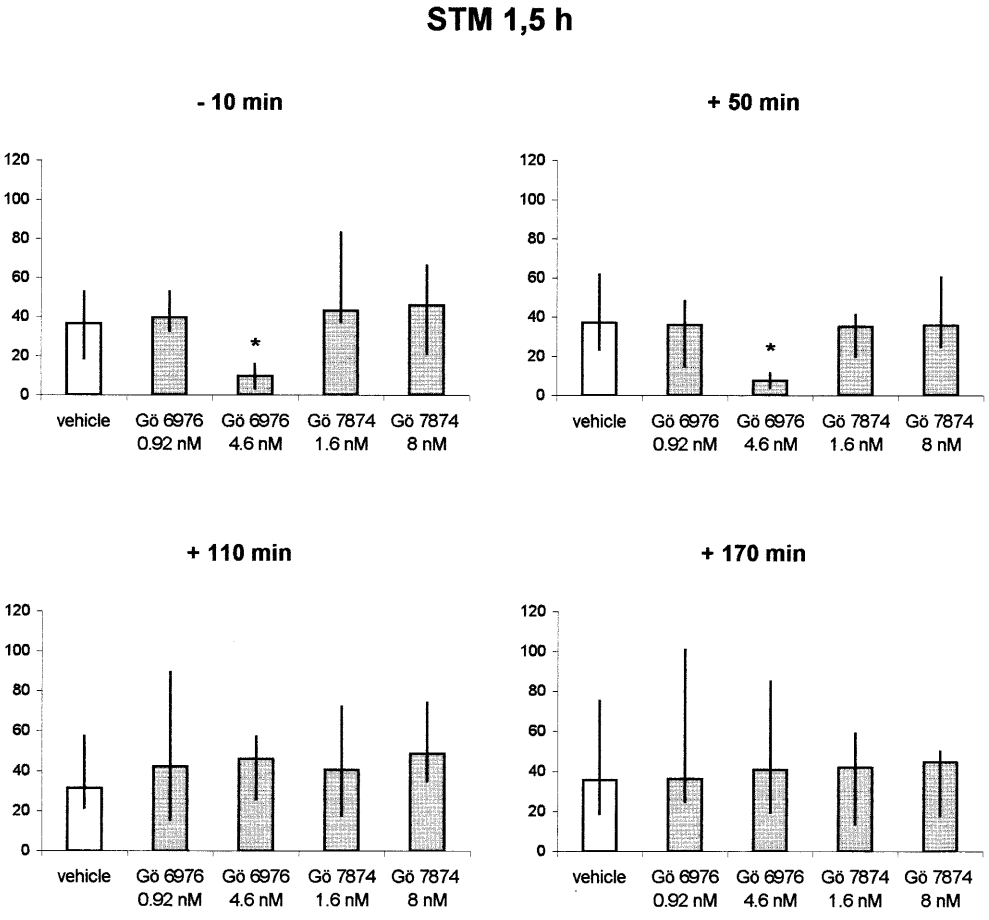

Fig. 1 In this and following figures, the *ordinates* represent test session step-down latencies expressed in seconds as medians (interquartile range), and the *abscissae* represent groups of rats infused into the dorsal CA1 region with vehicle, Gö 6976 (0.93 nM or 4.6 nM) or Gö 7874 (1.6 nM and 8 nM). This particular figure shows retention values obtained in short-term memory (STM) tests carried out 1.5 h after training (n=10 in the control groups and n=9–10 in the drug-treated groups). The *captions* indicate the time at which the treatments were given: 10 min prior to training (–10 min) or 50, 110, or 170 min after training. STM measured 1.5 h after training was inhibited by the higher concentration of Gö 6976 given 10 min before or 50 min after training, but not at other times, or by any of the other treatments. *Asterisks* indicate significant differences from controls at P<0.001 using a Mann-Whitney U test, two-tailed. Except for the group(s) marked with *asterisks*, training-test differences were significant at a P<0.02 level or less

the vehicle 10 min before or 50, 110, or 170 min after training (n=9–10 per group). The higher concentrations of each compound are close to their IC_{50} for α- and βI-PKC and for generic PKC, respectively (Paratcha et al. 2000). Test sessions were carried out 1.5, 3, and 24 h after training; the first two tests examined STM and the latter LTM (Izquierdo et al. 1998a, 1998b, 1998c, 1999; Vianna et al. 1999). As extensively described before (Izquierdo et al. 1998a, 1999), there was no tendency for retention test performance to increase or decrease with this type of repeated testing. In any case, in the present experiments there were no differences in retention test scores over the three tests in control animals (Fig. 1, Fig. 2, and Fig. 3). In test sessions, animals were placed on the platform, the latency to step down was measured up to a ceiling of 180 s, and no foot shock was given. Test step-down latencies were taken as a measure of retention. The use of a ceiling required analysis using non-parametric statistics (Kruskal-Wallis analyses of variance followed by Mann-Whitney U tests, two-tailed).

In the groups in which treatments were given 110 min or 170 min post-training and in which STM was tested at 3 h, in reality what was measured was STM retrieval rather than STM formation (Vianna et al. 1999; Walz et al. 1999). Clearly, the drugs did not influence the retrieval of STM. Experiment 2 studied the effect of the higher dose of each of the compounds on the retrieval of LTM. In this experiment, infusions were performed 10 min prior to the LTM test (n=10 per group). Since the drugs did inhibit LTM retention test performance (see below), it became necessary to investigate the effect of the two PKC inhibitors on exploratory activity in an open-field and on behavior in an elevated plus maze, such as is used to assess locomotor, anxiogenic (pro-conflict), or anxiolytic (anti-conflict) potential of drugs (Pellow et al. 1985; Novas et al. 1988). In these experiments, the number of animals used was nine in the open-field and ten in the plus maze in the control groups, and eight in the drug-treated groups. The open field was a 50-cm wide, 40-cm deep, 60-cm high wooden box with a linoleum floor subdivided into 12 equal rectangles by black lines and a frontal glass wall (Wolfman et al. 1999). The elevated plus maze was exactly as described by Pellow et al. (1985). The animals were exposed for 5 min to either the open-field or the plus maze, and received the vehicle or drug infusions 10 min before.

In both experiments, at the time of infusion, animals were gently handled and wrapped in a soft cloth. A 30-gauge infusion cannula attached to a microsyringe by a polyethylene tube was fitted into the guide cannulae, and the infusions were carried out for abut 1 min, first on one side and then on the other; the infusion cannula was left in place for an additional 15 s. The tip of the infusion cannula protruded 1 mm beyond that of the guide, reaching the pyramidal cell layer of CA1 in all cases. Histological controls were performed as described elsewhere, following a 0.5-µl infusion of 4% methylene blue through the same cannulae that had been used for vehicle or drug injection. This procedure gives a much more precise localization of where the infusion actually reached than the older method of merely locating the cannula tip (Martin 1991; Jerusalinsky et al. 1994; Ardenghi et al. 1997; Izquierdo et al. 1998c; Vianna et al. 1999; Walz et al. 1999). In all cases, diffusion of the dye – and therefore presumably of the drugs that had been given to the same animals 24 h before in the same sites – was less than 1 mm^3 (Martin 1991; Ardenghi et al. 1997). Only results from those animals with correct cannula placements (i.e., within 1 mm^3 of the intended site) were considered. In all experiments, the *Principles of laboratory animal care* (NIH publication No. 85–23, revised 1985) were followed.

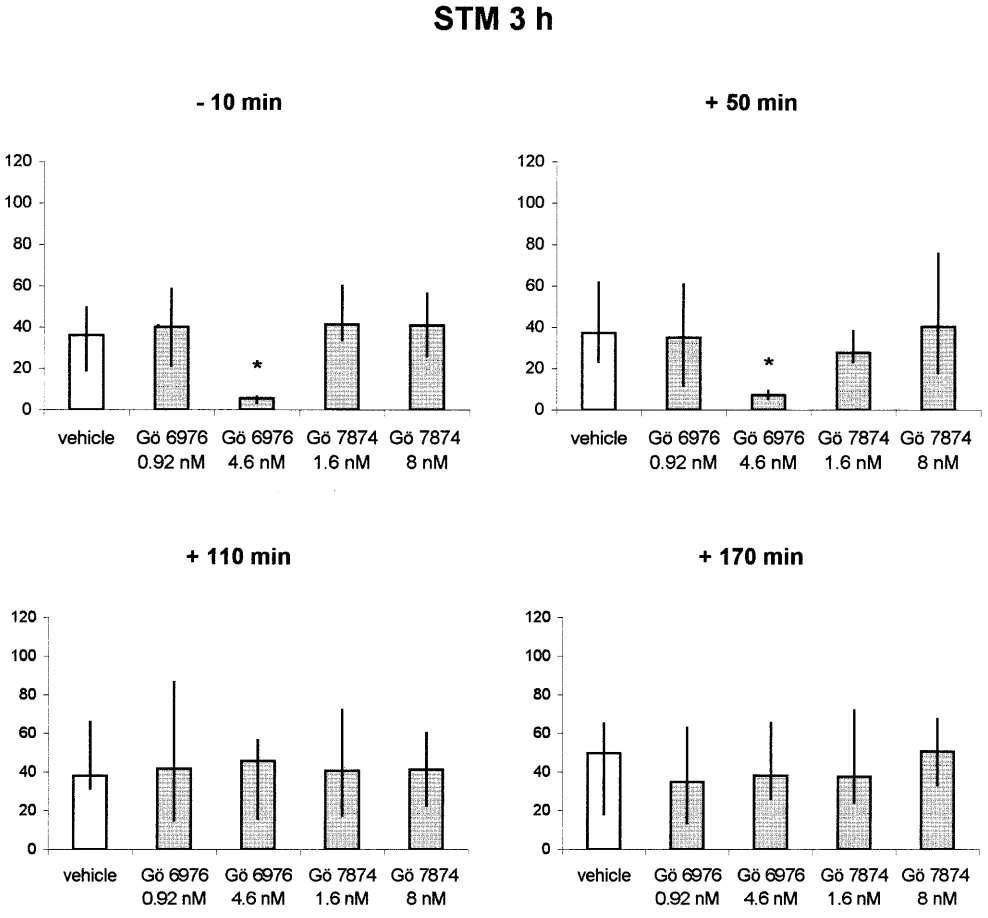

Fig. 2 Same as Fig. 1, but for short-term memory (STM) measured 3 h after training in the same animals. The results were similar: only the higher concentration of Gö 6976 given 10 min before or 50 min after training inhibited STM measured at 3 h

Results

Experiment 1

In this and the following experiment, training session latencies (overall mean 6.3, median 6.0, range 1.1–20.3, n=220) were not significantly different using a one-way analysis of variacne (ANOVA; F=0.8) or a Kruskal-Wallis test (H=1.79). The effects of the PKC inhibitors on STM are shown in Fig. 1, and those on LTM are shown in Fig. 2. There were significant group effects for the 1.5-h STM test (H=13.57, P=0.009), for the 3-h STM test (H=17.98, P<0.001), and for the LTM test (H=23.11, P<0.001).

In the STM tests, Gö 6976 at the 4.6 nM concentration but not at the 0.92 nM concentration produced amnesia when given 10 min before training or 50 min after training. Gö 7874 had no effect on STM at any of the two concentrations used (Fig. 1 and Fig. 2). In contrast, in the LTM test, both Gö 6976 and Gö 7874 caused amnesia at the highest dose used. The amnesia was complete when the drugs were given 10 min before or 50 min after training, and partial when given 110 min post-training. When administered 170 min after training neither PKC inhibitor had any effect (Fig. 3).

Experiment 2

The lack of effect of Gö 6976 and Gö 7874 given at 170 min on STM performance measured 10 min later (Fig. 1) indicates that the two substances do not affect the retrieval of STM. In experiment 2, we examined the effect of the intrahippocampal infusion of the higher concentration of the two drugs given 10 min prior to LTM testing at 24 h. Results are shown in Fig. 4. The two PKC inhibitors hindered LTM retrieval of this task.

In studies of this type, the possibility exists that animals may step down more quickly from the platform in the retrieval test because of a drug-induced enhancement of locomotor activity or a reduction of anxiety or fear. Thus, the influence of the higher dose of both compounds on crossings and rearings in the open field and on performance in the elevated plus maze were studied. Findings are shown in Fig. 5. Neither drug affected open-field performance. In the elevated plus maze, both compounds reduced the total number of entries and the number of rearings (suggesting a slight reduction of general activity in this test), and both reduced the number of entries in the open arms and showed a tendency of decreasing the time spent in the open arms suggesting a mild anxiogenic or pro-conflict effect.

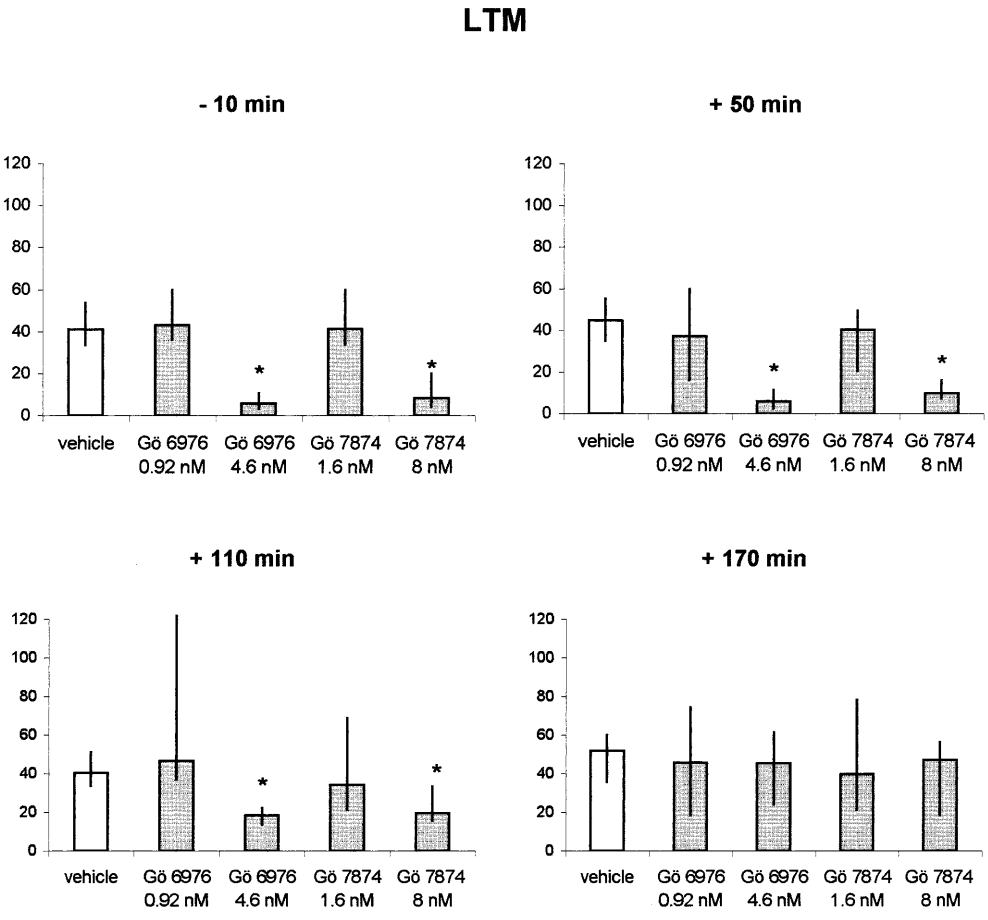

Fig. 3 Same as preceding figures, but for long-term memory (LTM) measured 24 h after training in the same animals. This was blocked by the higher concentration of both Gö 6976 and Gö 7864 given 10 min before or 50 min or 110 min after training. The effects on LTM of the treatments given at 110 min from training were slightly (P<0.05) but significantly less intense than those given at 50 min (Mann-Whitney U tests, two-tailed)

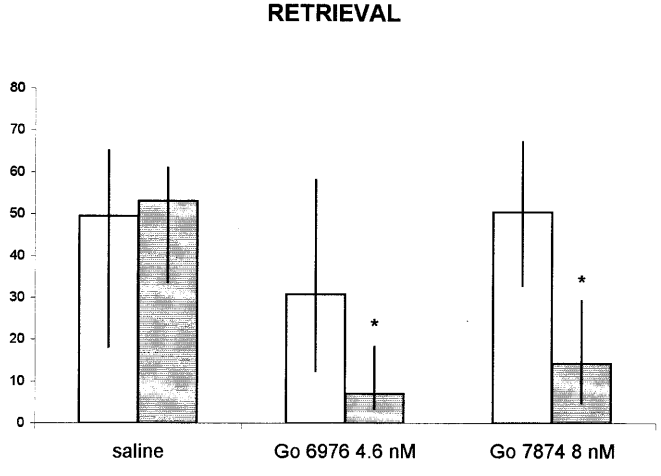

Fig. 4 Effects of vehicle (n=9), Gö 6976 (4.6 nM) and Gö 7874 (8 nM) (n=8 each) infused into the dorsal CA1 region 10 min prior to testing on retention test performance of the inhibitory avoidance task. The two drugs significantly disrupted retention test performance (P<0.002 using Mann-Whitney U tests, two-tailed)

Discussion

The findings on the effect of the two PKC inhibitors on LTM formation are very similar in nature and time course to those previously reported for the generic PKC inhibitors staurosporin and CGP 41231 (Jerusalinsky et al. 1994). They indicate that probably various isoforms of PKC participate in LTM formation, but show, in addition, that clearly α and/or βI are particularly important (Paratcha et al. 2000).

The findings on STM formation show that α- and/or βI-PKC, but not other isoforms of the enzyme, are crucial for this type of memory. This correlates with the report by Paratcha et al. (2000) showing a specific learning-induced increase of the activity of βI-PKC within the first few minutes after hours of inhibitory avoidance training. The findings on STM may also agree with those of Bourtchuladze et al. (1990) who described an amnesic effect of the unspecific PKC inhibitors mellitin and H7 on memory of inhibitory avoidance in the chick measured 3 h after training. Here, however, the generic inhibitor Gö 7874 had no effect on STM (Fig. 1), as was recently found to be the case with staurosporin (Izquierdo et al. 2000). Mellitin or H7 may act on other protein kinases, whereas Gö 7874 and, to an extent, staurosporin are more specific to PKC, although unspecific as to isoforms of the enzyme; which might dilute their effect if their influence on the α- and/or βI isoforms is proportionally lower than that on other isoforms.

The data of Fig. 1 clearly show that repeated testing over the first 3 h after training did not influence retention performance in successive tests. This agrees with several

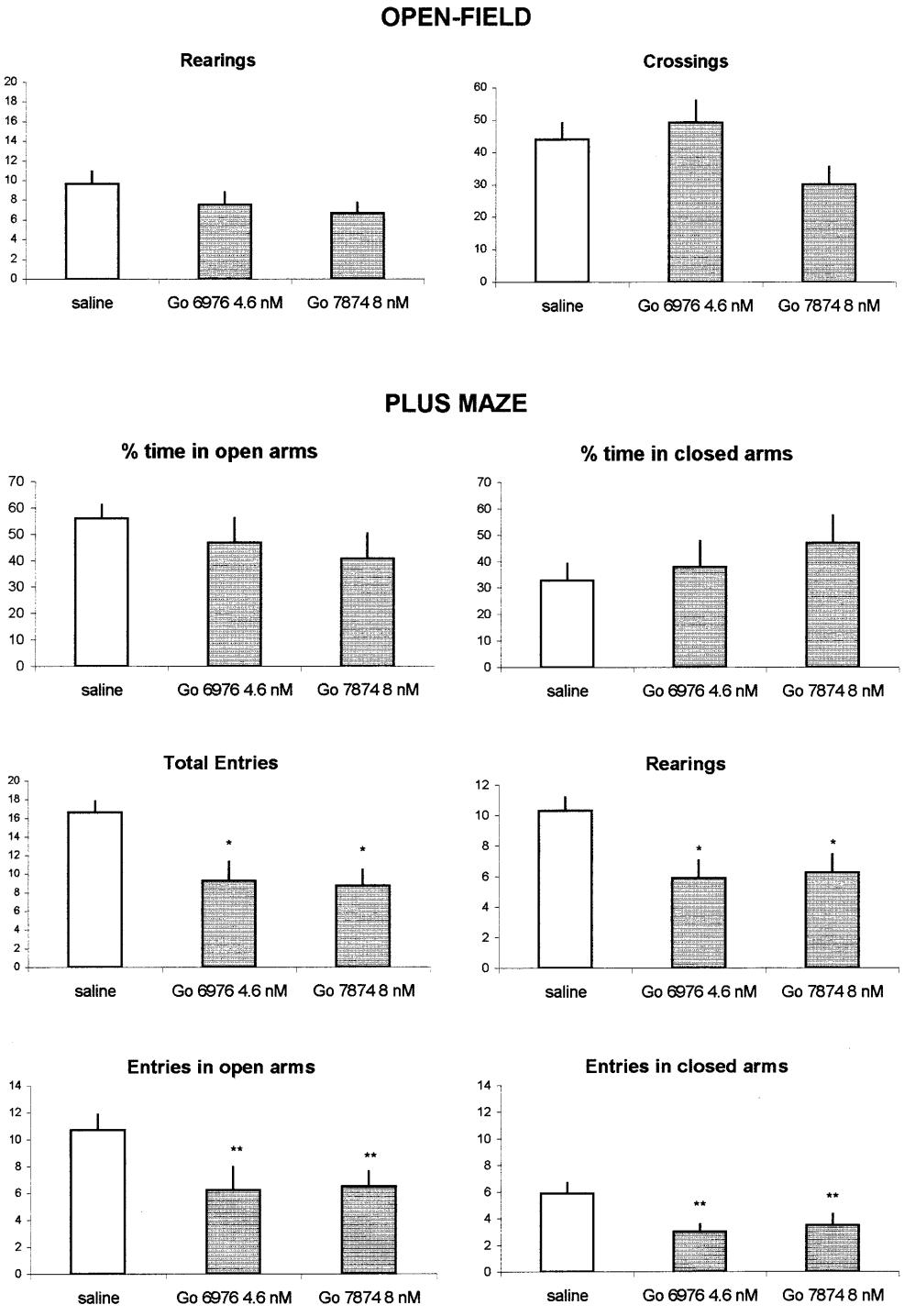

Fig. 5 *Upper*: Gö 6976 (4.6 nM) or Gö 7874 (8 nM) (n=8 each) infused into the dorsal CA1 region 10 min before exposure to the open-field for 5 min had no effect on rearings or crossings relative to that of the vehicle (n=9). *Lower*: the vehicle (n=10), Gö 6976 (4.6 nM) or Gö 7874 (8 nM) (n=8 each) were infused into the dorsal CA1 region 10 min before placing the animals for 5 min in an elevated plus maze. Neither drug had any effect on the percentage of time spent in either the open or the closed arms; but both drugs significantly reduced the total number of entries, the number of entries into either the open or the closed arms, and the number of rearings (*P<0.01; **P<0.02 using a Duncan test)

previous observations in untreated animals (Medina et al. 1999), in animals treated with saline or vehicle, and in rats that received numerous different post-training drugs treatments (Izquierdo et al. 1998a, 1999).

Aside from obviously providing yet another biochemical difference between the mechanisms of STM and those of LTM formation (Izquierdo et al. 1999; Vianna et al. 1999; Walz et al. 1999), the present findings raise several questions that require further research for an answer. First, is there a relationship between the involvement of the α and/or βI isoforms of PKC in STM and at the same time in LTM? Second, considering the separate deleterious effects of MAPK and protein kinase A inhibitors on STM and LTM (Vianna et al. 1999; Walz et al. 1999), what is the relationship of the present findings with those? There is considerable cross-talk between PKC, MAPK, and PKA cascades in neurons and other cells (Micheau and Riedel 1999), and this may be specific as to brain structure, or as to the physiological processes under study. The separate nature of STM and

LTM and the differential involvement of the hippocampus and other brain regions in each have been recently described in detail (Izquierdo et al. 1998c, 1999; Vianna et al. 1999).

The present findings provide, in addition, the first inkling on the possible molecular basis of LTM retrieval. Published findings so far have reported that retrieval of inhibitory avoidance (Izquierdo et al. 1993) and of spatial learning in the water maze (Vianna et al. 2000) require intact AMPA/kainate receptors in the hippocampus and elsewhere, at least within the first few days after training (Izquierdo and Medina 1997). Here, we show that the retrieval of LTM is dependent on PKC activity, but the retrieval of STM is not. Since the two inhibitors tested were effective on LTM retrieval, probably several isoforms of the enzyme are involved in it. The effect of the two compounds can certainly not be attributed to nonspecific behavioral influences (increased exploratory activity, decreased freezing behavior) on the LTM retrieval test.

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