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Changes in [^{3}H]glibenclamide binding to mouse forebrain membranes during morphine tolerance

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Abstract

The characteristics of specific binding of the ATP-sensitive K^+ (K_{ATP}) channel blocker [^{3}H]glibenclamide to forebrain membranes (P_2 fraction, 4°C) obtained from morphine-naive and -tolerant mice were evaluated. Morphine tolerance was induced by osmotic minipumps that released 45 mg/kg/day of morphine subcutaneously for 6 days. This treatment enhanced the antinociceptive ED_{50} of morphine without changing its E_{max}. In morphine-naive animals, (1) both the association and the dissociation of [^{3}H]glibenclamide were biphasic; (2) [^{3}H]glibenclamide was displaced by other sulfonylureas (order of potency: glibenclamide > glipizide ≫ tolbutamide) with pseudo-Hill coefficients lower than unity and biphasic Hofstee plots; and (3) Scatchard plots of saturation experiments were curvilinear, showed a Hill coefficient of 0.81 ± 0.04 and suggested the presence of two binding sites with a K_D of 0.13 and 3.17 nM and a B_{max} of 12.30 and 84.47 fmol/mg protein, respectively. By contrast, in membranes obtained from morphine-tolerant animals, (1) the Scatchard plots showed only one population of binding sites with a K_D of 0.87 nM and a B_{max} of 77.99 fmol/mg protein, and the Hill coefficient was very close to unity (0.96 ± 0.1); (2) competition experiments (using glibenclamide as displacer) showed a pseudo-Hill coefficient of 0.99 ± 0.04; and (3) dissociation experiments showed only one phase of dissociation. These results suggest that [^{3}H]glibenclamide binds to two different sites in membranes obtained from morphine-naive animals, but to only one site in morphine-tolerant animals. Consequently, it seems that morphine tolerance in mice involves adaptive changes in K_{ATP} channels. © 2001 Published by Elsevier Science B.V.

Keywords: Morphine tolerance; K^+ channel, ATP-sensitive; K_{ATP}; [^{3}H]Glibenclamide binding, Sulfonylurea; Forebrain membrane

1. Introduction

Ion channels (like membrane receptors) undergo adaptive modifications in their function or number during diseases processes (Gopalakrishnan and Triggle, 1990; Levin and Dunn-Meynell, 1998) or chronic drug treatments (e.g. Ferrante and Triggle, 1990). In particular, the chronic activation of voltage-dependent Na^+ channels (Shermann and Catterall, 1984), L-type Ca^{2+} channels (Skattebol et al., 1989) and ATP-sensitive K^+ (K_{ATP}) channels (Gopalakrishnan and Triggle, 1992) downregulates the number of each specific type of ion channel in the cell membrane. On the other hand, up regulation of these channels is induced by the chronic blockade of Na^+ channels (Brodie et al., 1989), L-type Ca^{2+} channels (Skattebol et al., 1989; Diaz et al., 1995) and K_{ATP} channels (Gopalakrishnan and Triggle, 1992; Lu et al., 1995).

The acute administration of μ-opioid receptor agonists opens inwardly rectifying K^+ (Kir) channels (Loose and Kelly, 1990; Chieng and Christie, 1994) and closes voltage-dependent Ca^{2+} channels (Kennedy and Henderson, 1991; Wilding et al., 1995). These electrophysiological effects appear to have behavioural consequences. For example, the antinociception induced by agonists of μ-opioid receptors is antagonized both by blockers of K_{ATP} channels (a type of Kir channel) (Ocaña et al., 1993a, 1995; Raffa and Martínez, 1995; Yang et al., 1998) and by L-type voltage-dependent Ca^{2+} channel openers (Dierssen et al., 1990; Wei et al., 1996), whereas it is enhanced by openers of K_{ATP} channels and by blockers of L- and N-type Ca^{2+} channels (Narita et al., 1992a; Ocaña et al., 1996; Del Pozo et al., 1990; Dierssen et al., 1990; Wei et al., 1996).

The chronic administration of agonists of μ-opioid receptors produces adaptive changes in the function and number of voltage-dependent Ca^{2+} channels, such as uncoupling of μ-opioid receptors and N-type Ca^{2+} channels (Kennedy and Henderson, 1991) and up regulation of L-type Ca^{2+} channels (Ramkumar and El-Fakahany, 1988;

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Diaz et al., 1995). The function of K^+ channels is also altered in morphine tolerance. Thus, the amplitude of the neuronal inwardly rectifying K^+ conductance induced by agonists of μ-opioid receptors is lower in animals rendered tolerant to morphine than in control animals (Christie et al., 1987; Zhang et al., 1996). Whether such functional changes reflect modifications in the properties or the number of K^+ channels is not well known. A previous study showed that chronic administration of morphine to rats downregulated the levels of mRNA for certain non-ATP-dependent K^+ channels (Mackler and Eberwine, 1994).

K_{ATP} channels are a type of Kir constituted by heteromultimers of two kinds of proteins. Each channel is formed from four pore-forming Kir subunits (Kir 6.1 or Kir 6.2) complexed with four regulatory sulfonylurea receptor proteins (SUR1 in neuronal/pancreatic beta cells or SUR2 in cardiovascular cells) (Inagaki et al., 1996; Aguilar-Bryan et al., 1998). Glibenclamide is a sulfonylurea drug which binds to the SUR1 domain with 10 to 500 fold higher affinity than to the SUR2 domains (Dorschner et al., 1999; Meyer et al., 1999). Experiments with [^{3}H]glibenclamide have demonstrated both the presence of specific binding sites (K_{ATP} channels) in neuronal membranes (Mourre et al., 1990; Gopalakrishnan et al., 1991a; Zini et al., 1991) and the development of adaptive changes in these binding sites during several diseases and after chronic treatment with K_{ATP} channel openers and blockers (e.g. Gopalakrishnan and Triggle, 1992; Lu et al., 1995; Levin and Dunn-Meynell, 1998).

We were interested in studying whether a sustained treatment with morphine changes the number or properties of K_{ATP} channels in the brain. Therefore, we evaluated the characteristics of [^{3}H]glibenclamide binding to neuronal membranes obtained from morphine-naive and -tolerant animals.

2. Materials and methods

2.1. Animals

Female Swiss CD1 mice weighing 28–30 g (Criffa, Spain) were used. Animals were kept in temperature-controlled rooms at 21 ± 1°C, with 12 h dark/light cycles (lights on at 0800 h and off at 2000 h) and free access to food and water. Antinociception experiments were performed between 0900 and 1300 h.

The animals were handled according to guidelines for the care of laboratory animals and ethical principles for research in experimental pain with conscious animals (Zimmermann, 1983).

2.2. Drugs and radioligands

[^{3}H]glibenclamide (specific activity 50.00 Ci/mmol) was purchased from Dupont-NEM (Itisa, Madrid, Spain). Dilutions were prepared in buffer (Tris 50 mM, pH 7.4). Unlabeled drugs were glibenclamide (Sigma Química, Spain); glipizide (Farmitalia Carlo Erba, Spain) and tolbutamide (Sigma Química). Glibenclamide and glipizide were dissolved in absolute ethanol to make up a 1-mM solution from which further dilutions were made with buffer (Tris 50 mM, pH 7.4). Tolbutamide was prepared in Tris 50 mM, pH 7.4, to make up a 25-mM solution (five drops of NaOH 0.1 N were added to dissolve the drug); further dilutions were prepared with Tris 50 mM, pH 7.4.

Morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Health Ministry) was used for antinociception studies and to induce morphine tolerance. It was dissolved in deionized water.

2.3. Experimental procedure

2.3.1. Experimental groups

Mice were treated with osmotic minipumps (Alzet 2001, Criffa, Spain) that released either morphine at a rate of 45 mg/kg/day (morphine-tolerant group) or its vehicle (morphine-naive or control group). The minipumps were implanted subcutaneously in animals anaesthetised with ethyl ether. Six days later, the animals, with the pump still implanted, were used for antinociception experiments or were killed and forebrain membranes were obtained as described below.

2.3.2. Membrane preparations

Binding experiments were carried out in the crude synaptosome fraction or P_2 fraction from mice forebrains. To obtain this fraction, we used the method of Zini et al. (1993). Briefly, mice were killed by decapitation, the brains were quickly removed and the forebrains were dissected and placed in tubes containing 10 ml ice-cold 0.32 M sucrose solution. Then each forebrain was homogenized with three strokes of Polytron homogenizer (Model PTA7T, Gomensoro, Spain) at a position of 3. Each stroke lasted 10 s and was separated from the next stroke by a 30-s period during which the tube was placed in ice. These homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C; the resulting pellets were discarded and the supernatants were recentrifuged under the same conditions. The resulting supernatants were poured off, taking care not to disturb the pellets, and were centrifuged at $17000 \times g$, for 20 min, at 4°C. Then each pellet (P_2 fraction) obtained was resuspended in buffer (Tris 50 mM, pH 7.4) and thoroughly mixed using a vortex.

Protein concentrations were measured by the method of Lowry et al. (1951) with some modifications, using bovine serum albumin as the standard.

2.3.3. Radioligand assays

We incubated 460 μl of crude synaptosome fraction with 20 μl of tritiated ligand and 20 μl of unlabeled drug or its solvent at 4°C. The incubation time was different depending on the type of assay. For protein, saturation and competition experiments we used 120 min, but in associa-

tion experiments, different times were used ranging from 0.5 to 180 min. In dissociation assays we incubated membranes and radioligand for 60 min; then unlabeled drug (1 μM) was added and bound [^{3}H]glibenclamide was measured at several time intervals ranging from 1 to 240 min. Incubations were terminated by adding 5 ml ice-cold 50 mM Tris–HCl buffer, pH 7.4, at 4 °C. Bound and free [^{3}H]glibenclamide were immediately separated by rapid filtration under a vacuum with a Brandel cell harvester (Model M-12T, Brandel Instruments, MD) over Whatman GF/B glass fibre filters, and washed twice with 5 ml volumes of ice-cold buffer. The filters were transferred to scintillation counting vials containing 4 ml of liquid scintillation cocktail (Optiphase Hisafe II, Wallac Scintillation Products, England) and equilibrated for at least 12 h. The radioactivity retained in the filter was measured with a liquid scintillation spectrometer (Beckman Instruments España S.A., Spain), with an efficiency of 52%.

The kinetics and competition studies were done in the presence of 2 nM [^{3}H]glibenclamide. In saturation studies, a range of [^{3}H]glibenclamide concentrations from 0.05 to 32 nM was used. Specific binding was defined as that displaced by 1 μM unlabeled glibenclamide.

All incubations were done in triplicate and each experiment was repeated three or more times to replicate the results.

2.3.4. Measurement of antinociception

Antinociception was assessed by the hot plate test at 50 ± 0.5°C as previously described (Robles et al., 1996). Six days after the minipump was implanted (with the pump still in place), morphine or its vehicle was injected subcutaneously (s.c.) in a volume of 5 ml/kg, 30 min before the animals were exposed to the hot plate. Then each mouse was placed on the surface of the hot plate and the latency to the beginning of jumping was recorded (the control latency value was 112.11 ± 6.67 s). The maximum time of exposure to the plate (cut-off time) was 500 s, and animals that did not jump after this time were removed from the plate and latency was recorded as 500 s. At the end of the experiments, all the animals were killed to avoid unnecessary suffering due to possible tissue lesions. Antinociceptive activity was expressed as the percentage of maximum possible antinociception, according to the equation: % antinociception = [(LTT − LTC)/(CT − LTC)] × 100, where LTT is the latency in morphine-treated mice, LTC is the latency in vehicle-treated mice, and CT is the cut-off time or maximum possible time on the plate.

2.4. Data analysis

Radioligand binding data were analysed using the non-linear least-squares analysis computer program EBDA/LIGAND (McPherson, 1985). This program performs Scatchard analyses (specific bound/free concentration of radioligand versus specific bound) and calculates the equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}). The program also fits data assuming the presence of one or more binding sites and allows comparison of the relative goodness-of-fit using a partial F test (the differences were considered significant when P was less than 0.05). We also performed Hill plots from the saturation experiments by plotting the data as log $[B/(B_{max} - B)]$ versus log [L], where B is the amount of radioligand bound at each concentration of radioligand [L] and the slope of the plot (n_H) represents the Hill coefficient.

Using the data from the competition experiments, the EBDA/LIGAND program provided the Hofstee analysis (% binding displaced versus % binding displaced/[displacer, nM]) and calculated a pseudo-Hill coefficient (n'_H) by plotting the data as log $[B'/(B'_{max} - B')]$ versus log [displacer, M]. In this case, B' is the specific binding in the presence of displacer, and B'_{max} represents the amount of specific binding in the absence of any concentration of displacer.

Association and dissociation experiments were analysed with the KINETIC computer program (McPherson, 1985). If the P was less than 0.05 (partial F test), the model for biphasic kinetics was considered more appropriate than a monophasic kinetics model. The association was described by the following equation:

$$B_t = B_{e1}(1 - e^{-k_{obs1}t}) + B_{e2}(1 - e^{-k_{obs2}t})$$

where B_t is the amount of radioligand bound at time t, B_e is the amount of radioligand bound at equilibrium at sites 1 and 2, respectively, and k_{obs1} and k_{obs2} are the apparent association rate constants at these sites.

The data obtained in dissociation experiments were fitted to the following equation:

$$B_t = B_{01}e^{-k_{-1A}t} + B_{02}e^{-k_{-1B}t}$$

where B_0 is the amount of radioligand bound at time 0 at the different sites, and k_{-1A} and k_{-1B} represent the dissociation rate constants for each phase.

The IC_{50} (concentration of unlabeled drug that inhibited 50% of specific [^{3}H]glibenclamide binding) and ED_{50} (dose of morphine that produced half of the maximal antinociception) values were calculated from the dose–response curves using non-linear regression analysis with the GraphPad Inplot computer program (Graph-Pad Software).

In the antinociception experiments, significant differences between two means were assessed by the Student's t-test and were accepted at the $P < 0.05$ level. Results are presented as mean ± S.E.M. value.

3. Results

3.1. Characteristics of [^{3}H]glibenclamide binding in control animals

Specific [^{3}H]glibenclamide binding was linear at protein concentrations between 0.4 and 2.2 mg/ml (Fig. 1A).

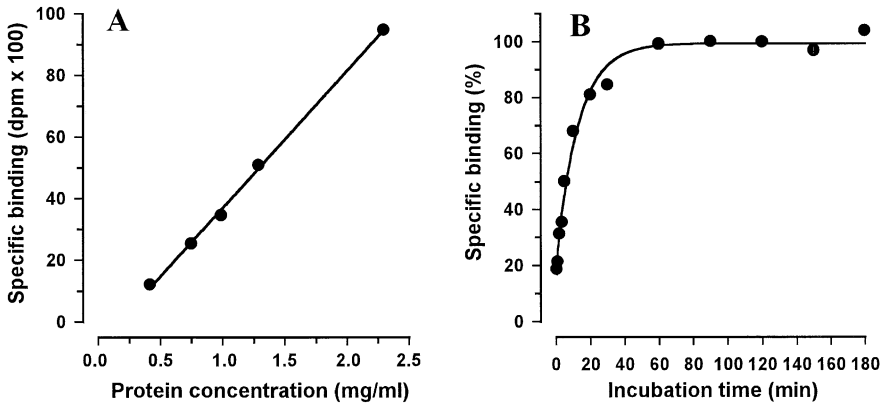

Fig. 1. Specific binding of [^{3}H]glibenclamide to morphine-naive mice forebrain membranes (P_2 fraction) as a function of protein concentration (A) and time of association (B). Membranes were incubated at 4°C with [^{3}H]glibenclamide 2 nM for 120 min (A) or for different periods (B). Specific binding was defined with unlabeled glibenclamide (1 μM), and represented more than 90% of the total binding. Data shown are representative of at least three experiments done in triplicate.

Binding experiments were therefore done at a final protein concentration of 0.8 mg/ml.

The time course of association of [^{3}H]glibenclamide was determined at a concentration of radioligand of 2 nM. Steady state was reached at 60 min and remained stable for 180 min (Fig. 1B). We therefore used 120 min as the incubation time for the rest of the experiments, except for dissociation experiments, for which we used a 60-min incubation period. Analysis of the time-course of the association indicated that a two-site model was significantly better ($P < 0.05$, F partial test) than a one-site model, the apparent association rate constants being $k_{obs1} = 0.091$ min^{-1} and $k_{obs2} = 0.669$ min^{-1}, respectively.

The dissociation of bound [^{3}H]glibenclamide also fit a bi-exponential model ($P < 0.001$, F partial test), with two dissociation rate constants: $k_{-1A} = 0.0049$ min^{-1} and $k_{-1B} = 0.3410$ min^{-1} (Fig. 2A).

Considering that $k_{+1} = (k_{obs} - k_{-1})/[F]$, we calculated the values of k_{+1} of the two sites, which were 0.043 and 0.161 min^{-1} nM^{-1} for k_{+1A} and k_{+1B}, respectively. The k_{-1}/k_{+1} ratios, a measure of the apparent dissociation constant, were 0.11 and 2.12 nM for K_{D1} and K_{D2}, respectively; these values were close to those obtained from saturation experiments (see below).

Saturation experiments showed that [^{3}H]glibenclamide bound in a saturable manner to the P_2 membranes (Fig. 3). The Scatchard analysis of these experiments yielded a biphasic (curvilinear) plot over the concentration range tested (Fig. 4A). Hill coefficients were different from unity (0.81 ± 0.04). LIGAND analysis showed that a two-site fit was significantly better than a one-site fit ($P < 0.01$). The equilibrium dissociation constants were 0.13 ± 0.04 nM (K_{D1}) for the higher affinity population and 3.17 ± 0.58 nM (K_{D2}) for the lower affinity, and the maximum num-

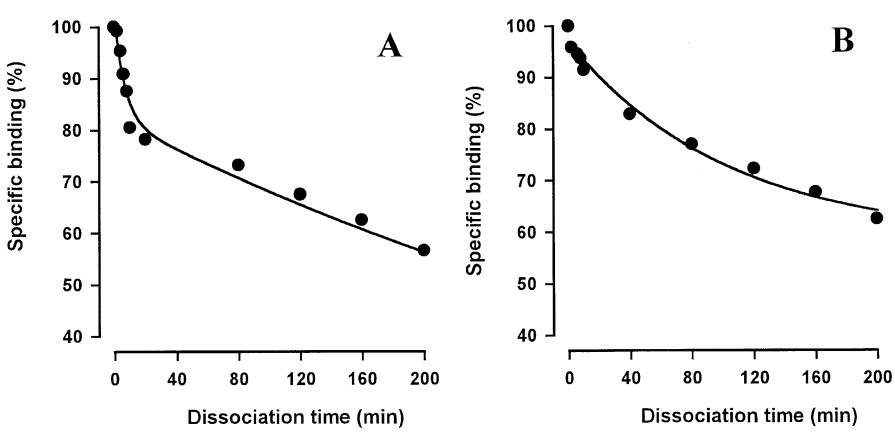

Fig. 2. Dissociation of [^{3}H]glibenclamide from forebrain membranes (P_2 fraction): (A) bi-exponential process of dissociation from morphine-naive mice membranes; (B) monoexponential process of dissociation from morphine-tolerant mice membranes. Dissociation was initiated by the addition of unlabeled glibenclamide (1 μM) to membranes equilibrated for 60 min at 4°C with [^{3}H]glibenclamide 2 nM, and was stopped by rapid filtration at the indicated times. Data shown are representative of at least three experiments done in triplicate.

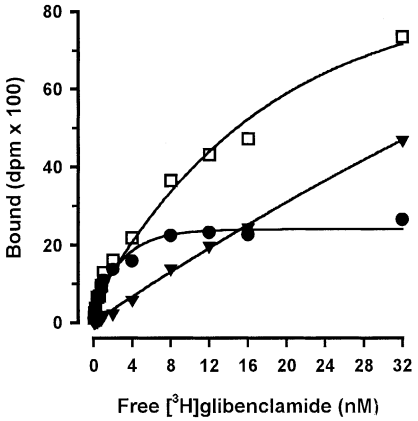

Fig. 3. Saturation analysis of [^{3}H]glibenclamide binding to control mice forebrain membranes (P_2 fraction). The total (□), specific (●) and non-specific (▼) binding were plotted as a function of free ligand concentration. Membrane proteins (0.8 mg/ml) and several concentrations (0.05–0.32 nM) of [^{3}H]glibenclamide were incubated with 1 μM unlabeled glibenclamide (non-specific binding) or its solvent (ethanol 0.1%) (total binding) for 120 min at 4°C. Specific binding was calculated as the difference between total and non-specific binding. Data shown are representative of at least three experiments done in triplicate.

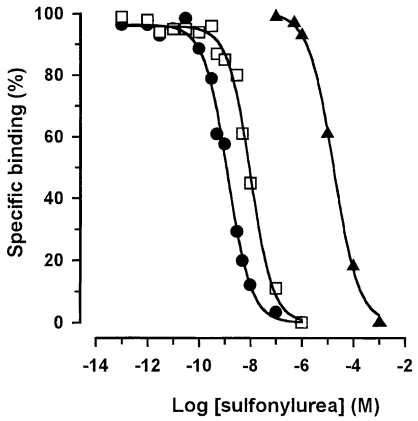

Fig. 5. Inhibition by unlabeled sulfonylureas of [^{3}H]glibenclamide specific binding to control mice forebrain membranes (P_2 fraction). [^{3}H]glibenclamide (2 nM) was incubated with 0.8 mg/ml membrane protein and increasing concentrations of glibenclamide (●), glipizide (□) or tolbutamide (▲) for 120 min at 4°C. Data shown are representative of at least three experiments done in triplicate.

ber (B_{max}) of specific [^{3}H]glibenclamide binding sites were 12.30 ± 4.56 and 84.47 ± 12.00 fmol/mg protein for B_{max1} and B_{max2}, respectively.

Competition experiments performed at equilibrium showed that all sulfonylureas tested (glibenclamide, glipizide and tolbutamide) concentration-dependently displaced specifically bound [^{3}H]glibenclamide (Fig. 5). The amount of specific binding was independent of the unlabeled sulfonylurea used, and it was always higher than 90% of the total binding. The order of potency of the different sulfonylureas in inhibiting [^{3}H]glibenclamide binding was glibenclamide > glipizide ≫ tolbutamide, and their inhibitory concentration 50 (IC_{50}) and 95% confidence interval were 1.59 (1.41–1.78) nM, 9.69 (7.78–12.06) nM and 16.78 (12.80–21.90) μM, respectively. The Hosftee analysis yielded a curvilinear plot in all cases, and the pseudo-Hill coefficients (n'_H) were always different from unity: 0.91 ± 0.05, 0.87 ± 0.08 and 0.85 ± 0.02 for glibenclamide, glipizide and tolbutamide competition, respectively.

3.2. Characteristics of [^{3}H]glibenclamide binding in morphine-tolerant animals

In the membranes obtained from morphine-tolerant animals, the Scatchard analysis of [^{3}H]glibenclamide saturation data showed only one population of receptors (Fig. 4B), with a K_D of 0.87 ± 0.20 nM, and a B_{max} value of 77.99 ± 5.08 fmol/mg protein. The slope of the Hill plot was not different from unity (0.96 ± 0.1). In addition, the pseudo-Hill coefficients obtained from competition experiments (using glibenclamide as the displacer) were very close to unity (n'_H = 0.99 ± 0.04) (data not shown). These results suggest the presence in morphine-tolerant animals of only one class of binding site with a K_D intermediate between that of the low- and high-affinity binding sites in

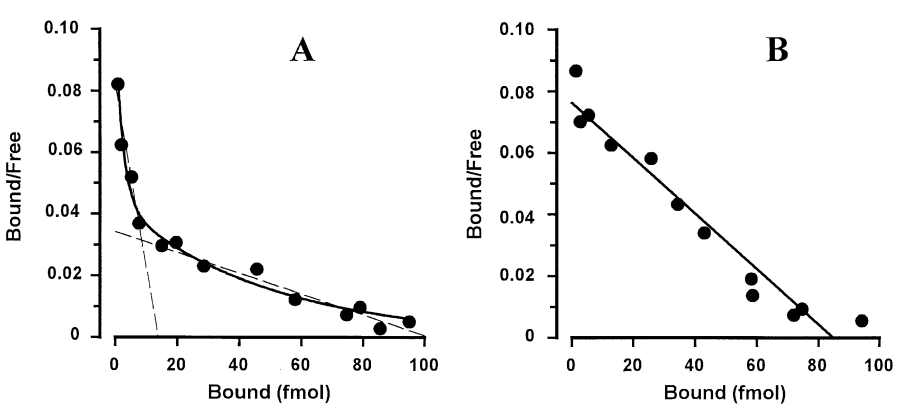

Fig. 4. Scatchard plots of [^{3}H]glibenclamide specific binding to forebrain membranes (P_2 fraction): (A) biphasic plot obtained from morphine-naive mice membranes; (B) monophasic plot obtained from morphine-tolerant mice membranes. Each figure is representative of the results obtained in four experiments done in triplicate. The dotted lines are estimates of the high- and low-affinity binding sites.

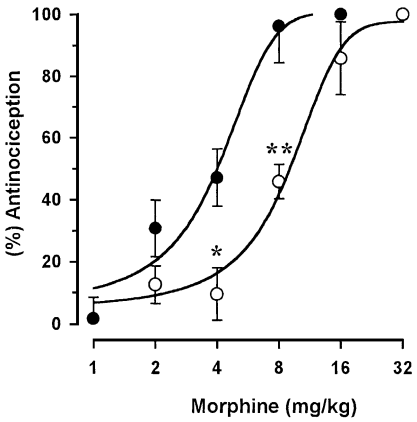

Fig. 6. Antinociception induced in the hot plate test (50±1°C, jumping response) by the subcutaneous administration of morphine (1–32 mg/kg) to animals implanted 6 days previously with osmotic minipumps that released either morphine 45 mg/kg/day (○) or its vehicle (●). The points and the vertical lines that cross them represent the mean±S.E.M. of the values obtained in a minimum of eight animals per dose. Statistically significant differences in comparison to control mice: $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

morphine-naive animals, and a B_{max} similar to that of the low-affinity site found in control animals.

Differences were also found between control and tolerant animals when dissociation experiments were analysed. In morphine-tolerant animals, the dissociation curve was monoexponential and the dissociation rate constant (k_{-1}) was 0.0023 min^{-1} (Fig. 2B).

3.3. Antinociceptive activity of morphine in control and morphine-tolerant animals

In mice implanted 6 days previously with minipumps that released vehicle, the administration of morphine (1–32 mg/kg, s.c.) induced a dose-dependent antinociceptive effect (Fig. 6). Maximum effect was reached with 16 mg/kg of morphine, and the ED_{50} was 4.08 ± 0.31 mg/kg. In mice implanted 6 days previously with minipumps that released morphine (45 mg/kg/day), the dose–response curve of morphine was shifted to right and the ED_{50} was 8.43 ± 1.01 mg/kg, a value significantly higher ($P < 0.01$) than in the control group (Fig. 6). There was no difference in the E_{max} of morphine between the two groups of animals (100.11 ± 3.56 in the control group versus 96.6 ± 6.5 in the morphine-tolerant group).

4. Discussion

The results we obtained with different experimental approaches (kinetic, competition and saturation experiments) showed that [^{3}H]glibenclamide recognizes two binding sites in morphine-naive animals but only one in morphine-tolerant animals. These results suggest that adaptive changes in the receptors for sulfonylureas (K_{ATP} channels) occur during the development of tolerance to morphine.

4.1. Characteristics of [^{3}H]glibenclamide binding in control animals

In the kinetics experiments, both the association and the dissociation of [^{3}H]glibenclamide to its binding sites were biphasic. A similar bi-exponential association and dissociation of [^{3}H]glibenclamide to neuronal and beta-cell membranes has been previously described (Panten et al., 1989; Zini et al., 1991; Schwanstecher et al., 1992). Scatchard analysis of the saturation isotherms in our control forebrain membranes yielded biphasic plots, and the Hill coefficients were less than unity; these facts suggest again that [^{3}H]glibenclamide binds to two different sites. Biphasic Scatchard plots have also been reported by others in neuronal membranes (Zini et al., 1991; Gopalakrishnan et al., 1991a; Niki and Ashcroft, 1993), as well as in insulin-secreting tumor (HIT) cells (French et al., 1991; Aguilar–Bryan et al., 1992; Nelson et al., 1992) and cardiac membranes (Gopalakrishnan et al., 1991a; French et al., 1991). The apparent K_D values obtained with the k_{-1}/k_{+1} ratios from the kinetics experiments were similar to those obtained with the Scatchard analysis, which supports the internal consistency of our data.

Competition experiments in membranes obtained from control animals showed that all sulfonylureas tested were equally able to displace [^{3}H]glibenclamide from its binding sites, the only difference being the order of potency as displacers: glibenclamide was one order of magnitude more potent than glipizide (IC_{50} for both in the low nM range), whereas tolbutamide was much less potent (IC_{50} in the μM range). These results agree with those obtained previously in brain membranes (Zini et al., 1991; Schwanstecher et al., 1994), pancreatic beta-cells (Panten et al., 1989) and HIT cells (Nelson et al., 1992). The results of competition experiments provided further evidence that [^{3}H]glibenclamide binds to two different sites, since the Hofstee plots were biphasic and the pseudo-Hill coefficients were lower than unity for all three sulfonylureas tested as displacers. Similar results have been described by Zini et al. (1991, 1993) in rat cerebral cortex membranes.

Therefore, experiments with forebrain membranes from our morphine-naive animals suggested that [^{3}H]glibenclamide binds to two different sites. The physiological significance of the high and low affinity binding sites is not clear, but both of them may play a functional role (Dunn-Meynell et al., 1997; Aguilar-Bryan et al., 1992; Nelson et al., 1992) and autoradiographic studies suggest that they are distributed differently in several areas of the brain and within the neurons of the same area (Dunn-Meynell et al., 1997). Furthermore, high- and low-affinity [^{3}H]glibenclamide binding sites have been shown to be differentially regulated by streptozotocin-induced diabetes

(Levin and Dunn-Meynell, 1998) as well as by dietary composition and by the propensity of the animals to develop or resist dietary obesity (Levin and Dunn-Meynell, 1997).

4.2. Characteristics of [^{3}H]glibenclamide binding in morphine-tolerant animals

The enhanced ED_{50} of morphine to produce antinociception in animals implanted with minipumps that released morphine in comparison to those implanted with vehicle-filled minipumps indicates that the former animals were in fact tolerant to morphine. The degree of tolerance induced by the dose of morphine released by the minipumps in our experiments was not very high: the ratio between the antinociceptive ED_{50} of morphine in the morphine-treated and -naive group was only 2.07. Interestingly, when the animals were made tolerant to morphine the characteristics of [^{3}H]glibenclamide binding to forebrain membranes were altered. In morphine-tolerant animals, the dissociation of [^{3}H]glibenclamide from its binding sites was monoexponential, the Scatchard analysis of saturation experiments yielded a linear plot, and the competition experiments yielded a linear Hofstee plot and a pseudo-Hill coefficient close to unity. These results indicate that during morphine tolerance there is only one [^{3}H]glibenclamide binding site with a B_{max} similar to that of low-affinity sites in control animals, and a K_D intermediate between that of the high- and low-affinity binding sites in morphine-naive animals. The modulation by morphine of [^{3}H]glibenclamide binding cannot be explained by a direct effect of morphine on the sulfonylurea receptor, since morphine is not able to displace [^{3}H]glibenclamide from its binding sites in neuronal membranes (Narita et al., 1992a; Raffa and Codd, 1994). The changes in the binding characteristics of [^{3}H]glibenclamide that we observed in the morphine-tolerant animals can be explained in part by two facts. Firstly, during morphine tolerance, the activity of some proteinkinases and the degree of phosphorylation of several proteins are enhanced (Nestler and Aghajanian, 1997; Nestler et al., 1999). Moreover, phosphorylation of the sulfonylurea receptor reduces the B_{max} of the high-affinity binding site of [^{3}H]glibenclamide in the hamster B-cell line HIT-T15 cells (Schwanstecher et al. 1992) and enhances the K_D value for [^{3}H]glibenclamide binding to the high-affinity site in HIT-T15 cells (Schwanstecher et al. 1992) and to microsomes obtained from mouse pancreatic islets (Schwanstecher et al. 1991). Therefore, a phosphorylation of the brain sulfonylurea receptor induced by the sustained administration of morphine may underlie the modification of the high-affinity [^{3}H]glibenclamide binding site characteristics that we found.

The changes in [^{3}H]glibenclamide binding as a result of chronic treatment with morphine are not surprising, since previous experiments have shown that [^{3}H]glibenclamide binding sites undergo adaptive changes as a consequence of chronic drug treatment or disease. Chronic treatment with glibenclamide up regulates [^{3}H]glibenclamide binding in cardiac and brain membranes (Gopalakrishnan and Triggle, 1992; Lu et al., 1995), whereas chronic administration of pinacidil downregulates binding in brain membranes (Gopalakrishnan and Triggle, 1992). Furthermore, changes in [^{3}H]glibenclamide binding to brain membranes were also observed during experimental diabetes (Levin and Dunn-Meynell, 1998), cardiac failure (Gopalakrishnan et al., 1991b) and in cardiomyopathic animals (Miller et al., 1992), as well as in animals subjected to hypoxia (Mourre et al., 1990; Xia et al., 1993).

One previous study reported an increase in the K_D and in the B_{max} of [^{3}H]glibenclamide to brain membranes during morphine tolerance (Welch et al., 1997). The changes in B_{max} were opposite to those we found. Although we do not known the reasons for the discrepancy, methodological differences may be partly responsible. Welch et al. used synaptosomes obtained from the whole brain of Swiss Webster mice treated for 3 days with morphine; whereas we used P_2 membranes obtained from the forebrain of CD-1 mice treated for 6 days with morphine. More importantly, Welch et al. found a single [^{3}H]glibenclamide binding site in control and morphine-tolerant animals. These results differ from those of several studies which found two binding sites for [^{3}H]glibenclamide in neuronal membranes of control animals (Zini et al., 1991; Gopalakrishnan et al., 1991a; Niki and Ashcroft, 1993; present results). Therefore, the experimental conditions used by Welch et al. may have precluded the identification of binding sites that were downregulated in our experiments.

Morphine is not able to directly bind to the Kir channels to modulate their activity (Ulens et al., 1999), but it is able to open Kir channels by binding to μ-opioid receptors and activating Gi proteins (Tatsumi et al., 1990, Chen and Yu, 1994). The opening of Kir channels, particularly K_{ATP} channels, appears to play a role in both the centrally and peripherally mediated acute effects of morphine. Morphine-induced antinociception is antagonized by the i.c.v. administration of several K_{ATP} channel blockers (Ocaña et al., 1990, 1995; Raffa and Martínez, 1995), whereas it is enhanced by K_{ATP} channel openers (Narita et al., 1993; Ocaña et al., 1996). Furthermore, K_{ATP} channel blockers are also able to antagonize other centrally mediated acute effects of morphine, such as hyperthermia (Narita et al., 1992b), hypermotility (Ocaña et al., 1993b) and memory impairments (Stefani et al., 1999). In addition, some peripheral effects of morphine, such as the cardioprotective effects of morphine against ischemia (Schultz et al., 1996; Liang and Gross, 1999) and the inhibitory effect of morphine on gall bladder emptying (Patil and Thakker, 1996) are also antagonized by K_{ATP} channel blockers.

Because the opening of Kir channels seems to play a role in the acute effects of morphine, modifications in the function or number of Kir channels would be expected as

part of the adaptive changes that take place during morphine tolerance. In fact, sustained exposure in vitro to high concentrations of μ-opioid receptor agonists favors desensitization of the inwardly rectifying K^+ currents induced by the μ-opioid agonist (Chen and Yu, 1994; Osborne and Williams, 1995; Kovoor et al., 1995). Furthermore, in animals made tolerant to morphine in vivo, the ability of μ-opioid receptor agonists to activate neuronal inwardly rectifying K^+ conductances was impaired (Christie et al., 1987; Zhang et al., 1996). Our results indicate that such changes might be due, at least in part, to modifications in the characteristics of the constitutive protein of K_{ATP} channels, i.e. downregulation of the high affinity state for [^{3}H]glibenclamide, in morphine-tolerant animals.

In conclusion, our results together with those previously published by others, obtained with electrophysiological and molecular biology techniques, suggest that a change in the function and number of K^+ channels is one of the adaptive processes that occurs during the repeated administration of morphine.

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