NOTES

In Vitro Model for Studying Effects of Morphine and Nalorphine on ^{45}Ca–Ganglioside Binding

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Abstract □ The characteristics of ^{45}Ca–ganglioside (bovine) binding and the effects of morphine and the analgesic narcotic antagonist nalorphine on ^{45}Ca–ganglioside binding were evaluated. Morphine, in concentrations equivalent to that found in the brain during analgesia, decreased the binding of ^{45}Ca to purified bovine gangliosides. Nalorphine exhibited a biphasic action on ^{45}Ca–ganglioside binding. In low concentrations, nalorphine enhanced radiocalcium binding to gangliosides while high concentrations antagonized ^{45}Ca binding. Nalorphine partially antagonized morphine-induced inhibition of ^{45}Ca–ganglioside binding. The results presented demonstrate that morphine alters the binding of calcium to a neuronal specific glycophospholipid, the ganglioside. An *in vitro* model for studying morphine and nalorphine interactions with calcium binding is presented.

Keyphrases □ Calcium (radiolabeled) binding to *in vitro* bovine gangliosides—effects of morphine and nalorphine □ ^{45}Ca binding to *in vitro* bovine gangliosides—effects of morphine and nalorphine □ Nalorphine—effect on *in vitro* ^{45}Ca binding to bovine gangliosides □ Morphine—effect on *in vitro* ^{45}Ca binding to bovine gangliosides □ Gangliosides (bovine)—*in vitro* binding of radiocalcium, effects of morphine and nalorphine

Kakunga *et al.* (1), Mulé (2), and other investigators reported that calcium ions antagonize the analgesic effects of morphine and that morphine and other narcotic analgesics affect radiocalcium phospholipid binding. These investigators postulated that the narcotic analgesics compete with calcium for anionic binding sites on phospholipid molecules in the neuronal membrane. Displacement of calcium from membrane binding sites would lead to changes in membrane permeability to other ions and result in alterations in electrolyte distribution within the neuron.

Gangliosides are a group of acidic glycolipids found in membrane fractions of the cerebral cortex, medulla oblongata, thalamus, and other areas of the nervous system (3–5). Gangliosides are functional membrane constituents and do not merely contribute to the structural conformation of the neuronal membrane (4). The electrical excitability of neurons can be inhibited by neutralizing their anionic sites with protamine. Restoration of neuronal electrical excitability occurs after the addition of gangliosides. Gangliosides have been postulated to be the ionic binding sites of neuronal membranes (6). In addition, gangliosides may partake in other neuronal functions; Duel *et al.* (7) presented evidence suggesting that serotonin storage sites in the brain may be a serotonin–metallic ion–ganglioside–energy complex. Morphine has been shown to release serotonin *in vivo* (8, 9). The ability of morphine to alter calcium binding and possibly to release serotonin may be related to an interaction of morphine with gangliosides.

A simple direct method to test the hypothesis that morphine may affect the calcium binding characteristics of gangliosides is to use commercially available gangliosides and determine the effect of morphine on the ability of this substance to bind ^{45}Ca.

EXPERIMENTAL

The methods used were similar to those described by Feinstein (10) and Quarles and Folch-Pi (11). Purified bovine gangliosides[1] were dissolved in one volume of distilled water to which 19 volumes of chloroform–methanol (2:1 v/v) were added to give a final ganglioside concentration of 1 mg./ml. Ten-milliliter aliquots of gangliosides were added to test tubes containing 2 ml. of 1, 3, or 5 mM $CaCl_2$ and $^{45}CaCl_2$ (0.1 mc./mmole/l.) in distilled water. The tubes were mixed for 1 min. and centrifuged at 2500 r.p.m. for 10 min. This provided an upper aqueous phase and lower organic phase. Aliquots (1.0 ml.) from each phase were placed in planchets and evaporated overnight. The radioactivity in each phase was determined on a gas flow counter[2] at constant self-absorption. The experiments were repeated with the addition of morphine hydrochloride (2.5 and 5.0 mcg.), nalorphine (2.5 and 5.0 mcg.), and the combination of drugs to the test tube (in a volume not exceeding 0.01 ml.) prior to mixing. In all experiments, a blank solution containing only chloroform–methanol–water (6:3:1) was utilized. The data represent the quantity of radiocalcium bound to the gangliosides in the organic phase and were compared with Student's paired t test (12). All data are expressed as counts per minute per milliliter of ^{45}Ca per milligram of ganglioside.

RESULTS AND DISCUSSION

The system described by Quarles and Folch-Pi (11) was utilized rather than a solution containing physiologic concentrations of other ions because of the reported interaction of sodium, potassium, and perhaps magnesium with calcium–ganglioside binding. Any change observed could not have been attributed to a direct effect of morphine on ^{45}Ca-binding but may have been due to alterations in any of the other ions as well. It was decided, therefore, to eliminate this possibility and to test directly the effects of morphine on ^{45}Ca–ganglioside binding with only morphine, calcium, and ganglioside present. This technique was also used by Mulé (2). The same reasoning held for not using physiological pH. The concentrations of buffer (phosphate or tromethamine) could interact in this system.

In the absence of gangliosides, ^{45}Ca was present only in the aqueous phase. When gangliosides were present in the solution, radioactivity was found in the organic phase (Figs. 1A and 1B). The uptake of tracer by the gangliosides was directly related to the concentration of calcium in the aqueous phase. Approximately 5%

[1] Sigma Co., type III purified.
[2] Beckman Wide Beta.

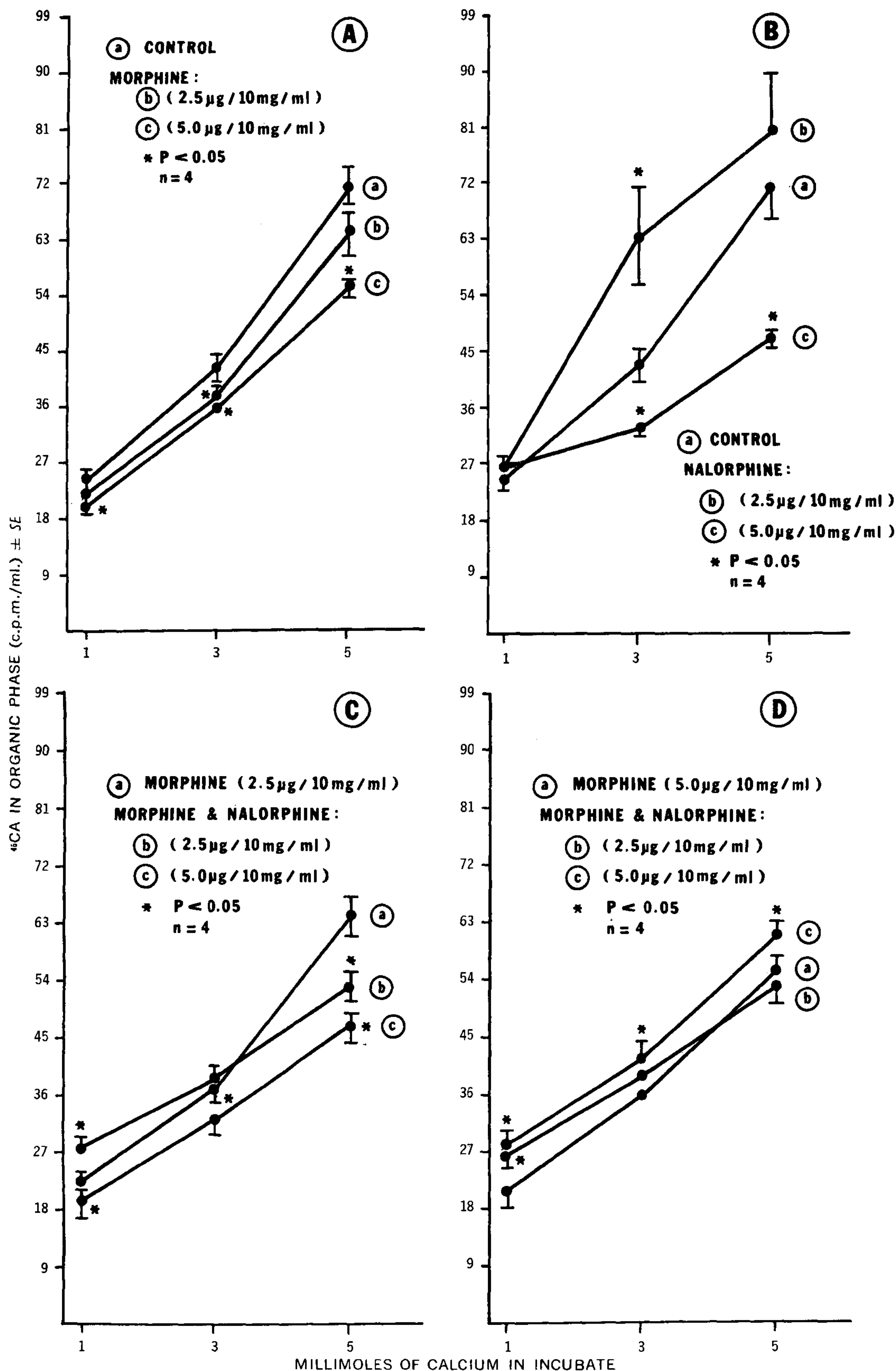

Figure 1—*Binding of radiocalcium by gangliosides in the presence of: (A) morphine, (B) nalorphine, (C) low concentrations of morphine in the presence of high and low concentrations of nalorphine, and (D) high concentrations of morphine in the presence of high and low concentrations of nalorphine (D). Each point represents the mean of four experiments. Each vertical bar denotes the standard error of the mean. An asterisk denotes that the means differ significantly* ($p < 0.05$) *from the corresponding control.*

of the radiocalcium was bound to gangliosides, demonstrating that the equilibrium appears to be in favor of the unbound or dissociated calcium ion. These results confirm the finding of Quarles and Folch-Pi (11) that gangliosides can combine with calcium, at physiological calcium concentrations, to form a water-insoluble stable complex.

The negatively charged carboxyl groups of the sialic acid residues (5) of the glycolipid gangliosides bind univalent and divalent cations. Electrical excitability of neurons can be blocked after the addition of positively charged substances, such as protamine, to brain preparations. Electrical activity is restored upon the addition of gangliosides. The effects of morphine appear to be dependent upon the presence of intact membranes. Therefore, many investigators have postulated that morphine interacts with the cell membrane to alter the binding of calcium to membrane binding sites. Experimental evidence has demonstrated that narcotic analgesics will, in fact, alter the binding of calcium to phospholipid fractions of cell homogenates (2). However, to our knowledge, evidence demonstrating an effect of morphine on the binding of calcium to a neuronal specific phospholipid has not been reported previously.

The data presented in Fig. 1A demonstrate that morphine reduced the binding of ^{45}Ca to neuronal specific glycolipid gangliosides. This effect was concentration related. Nalorphine, an analgesic narcotic antagonist, exerted a biphasic effect on radiocalcium–ganglioside binding (Fig. 1B). This effect was only significant at a measurable calcium concentration of 3 mmoles. Nalorphine in low concentrations enhanced ^{45}Ca binding to gangliosides, yet high concentrations depressed the calcium binding capacity of bovine gangliosides. If, as many investigators have postulated, the analgesic effect of narcotic analgesics is related to effects on ion binding, one would not be surprised to encounter a biphasic effect with an analgesic narcotic antagonist.

The effects of nalorphine in combination with morphine on ^{45}Ca–ganglioside binding are more difficult to interpret. If one postulates that morphine competitively displaces calcium from binding sites on gangliosides and nalorphine enhances binding, then the observable effects should be the algebraic sum of the enhanced and depressed binding. This is not observed. A low concentration of nalorphine with respect to morphine (curve b of Fig. 1D) does not affect significantly ($p > 0.05$) the ability of morphine to depress ^{45}Ca–ganglioside binding at physiological calcium concentrations. Equal concentrations of nalorphine with respect to morphine do not either affect or antagonize morphine-induced depression of ^{45}Ca–ganglioside binding (curve b, Fig. 1C, and curve c, Fig. 1D). However, a high concentration of nalorphine with respect to morphine potentiates the ability of morphine to decrease ^{45}Ca–ganglioside binding (curve c, Fig. 1C). These results suggest that the interaction of morphine and nalorphine wth ^{45}Ca–ganglioside binding is dependent not only on the relative concentrations of each narcotic but also on the total concentration of narcotic and antagonist within the system studied.

As stated, these results are difficult to interpret. However, if one postulates that the analgesic action of morphine is related to its ability to alter calcium binding to neuronal constituents, these data can be interpreted as suggesting that narcotic antagonist reversal may be more complex than merely antagonist displacement of morphine from binding sites. A second possibility is that the effect of morphine on ^{45}Ca binding may not be related to its analgesic action. Inhibition of calcium transport from aqueous to organic phases is a common property of many centrally acting drugs (2). If, in fact, inhibition of facilitated calcium transport is only partly or indirectly related to the analgesic effects of morphine and other centrally acting agents, then to what action of these compounds could one ascribe the effects on ^{45}Ca binding? Morphine, as well as other centrally acting drugs, has been demonstrated to release serotonin and other transmitters *in vivo* and *in vitro* (8, 9). Alterations in the binding of calcium to membranes could ultimately alter the release of neurotransmitters. Therefore, decreased calcium binding or transport could represent the serotonin or biogenic amine releasing properties of morphine which could induce, modify, or have no relation to the analgesic effects of morphine.

The data presented in this report cannot resolve the postulates concerning the effect of morphine on ^{45}Ca binding. However, the data do demonstrate, for the first time, the ability of morphine to depress ^{45}Ca binding to a neuronal specific glycophospholipid, the ganglioside. Studies that measure the calcium binding characteristics of endogenous gangliosides in the presence and absence of morphine analgesia must be performed if the significance of this effect is to be ascertained. Our laboratory is not equipped to perform ganglioside isolation. In writing this short note, it is our intention and hope that some investigator in the field may utilize our suggestions and determine whether or not this reported effect of morphine with ^{45}Ca–ganglioside binding has a pharmacological counterpart *in vivo*.

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