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Antinociception Following Microinjection of Dibutyryl Cyclic Nucleotides into the Caudal Reticular Formation and Periaqueductal Gray of the Rat Brain

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LEVY, R. A., H. K. PROUDFIT AND B. D. GOLDSTEIN. *Antinociception following microinjection of dibutyryl cyclic nucleotides into the caudal reticular formation and periaqueductal gray of the rat brain.* PHARMACOL BIOCHEM BEHAV **19**(1) 79–84, 1983.—The tail flick, paw pinch, and hot plate tests were used to assess changes in nociceptive threshold following microinjection of dibutyryl derivatives of cyclic nucleotides into areas of the central nervous system previously shown to be involved in modulation of nociceptive threshold and mediation of morphine analgesia. An elevation in the nociceptive threshold was observed on all three tests following administration of 10 μg dibutyryl cyclic 3′:5′ adenosine monophosphate (db cAMP) into the caudal brainstem reticular formation (CRF) and periaqueductal gray (PAG). Two μg db cAMP produced the same magnitude of analgesia but had a shorter duration of action. Twenty μg dibutyryl cyclic 3′:5′ guanosine monophosphate (db cGMP) produced analgesia on all three tests following microinjection at CRF sites but not at PAG sites. These data indicate that morphine analgesia and the antinociception produced by cyclic nucleotides may involve, at least in part, common neuronal substrates. However, the observed capacity of db cAMP to elevate nociceptive threshold does not support the hypothesis that the mechanism of morphine's analgesic action involves inhibition of adenylate cyclase.

Antinociception Analgesia Cyclic nucleotides Caudal reticular formation Periaqueductal gray

CYCLIC guanosine and adenosine nucleotides have been shown to produce antinociception following intracerebroventricular administration in rodents [1, 5, 28]. Since these agents may act as second messengers or modulate transmission at cholinergic and monoaminergic junctions [4, 20, 23], this antinociception may reflect an action at such synapses in nuclei which regulate pain threshold. The reticular formation of the caudal brainstem (CRF) and the periaqueductal gray of the mid-brain (PAG) are both involved in the control of nociceptive threshold [8, 17, 19, 29], and there is some evidence that neurons in both structures receive cholinergic and noradrenergic innervation (see Discussion). It is possible, therefore, that at least part of the antinociception produced following intracerebroventricular administration of cyclic nucleotides may reflect a direct action on neurons in either of these two structures. In the present study, we have investigated the actions of dibutyryl guanosine and adenosine cyclic nucleotides on neurons in the CRF and PAG by measuring changes in pain sensitivity following local application of these agents by microinjection. A preliminary account of these findings has been published [16].

METHOD

Cannula Implantation

Stainless steel guide sheaths (22 gauge) were implanted in Sprague-Dawley or Holtzman rats (either sex, 250–450 g) under pentobarbital anesthesia. The sheath was stereotaxically positioned about 3.0 mm above the target area in the caudal brainstem reticular formation (CRF) or mid-brain periaqueductal gray (PAG). The CRF coordinates were P 2.5–3.0, H −1.0, L 0.0 or 1.0; incisor bar −2.5 mm. The PAG coordinates were A 0.6–0.7, H −4.0, L 0.7; incisor bar −2.5. The guide sheath was fitted with a 28 gauge stylet to prevent occlusion and attached to the skull with jeweler's screws and dental acrylic. Animals were housed individually after surgery and allowed at least a week to recover.

Microinjection Procedure

Drugs were microinjected at the target sites using a 28 gauge stainless steel injection cannula inserted through and extending about 3.0 mm beyond the guide sheath. All drugs

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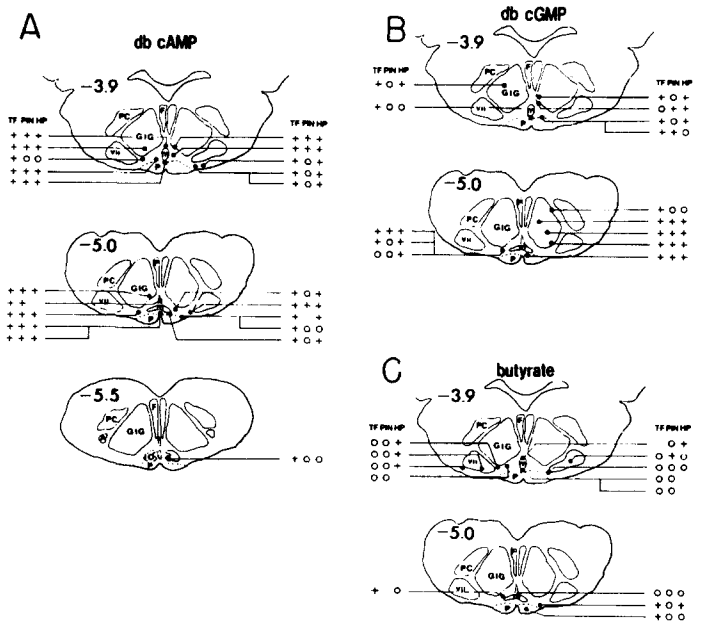

FIG. 1. Alteration in nociceptive threshold following microinjection of db cAMP (10 μg), db cGMP (20 μg), and butyrate (10 μg) at sites in the caudal reticular formation. Antinociception (+), or lack thereof (o), following administration at a given site was assessed on the tail flick (TF), paw pinch (PIN), and hot plate (HP) tests. See text for details. GIG, nucleus reticularis gigantocellularis; PC, nucleus reticularis parvocellularis; m, nucleus raphe magnus; F, median longitudinal fasciculus; VII, nucleus of the seventh nerve; P, pyramidal tract; 10, inferior olive; A, nucleus ambiguous. The negative numbers in each coronal section indicate the distance (mm) caudal to the interaural line.

were delivered in a fixed volume (0.5 μl) of 0.9% NaCl over a period of about one minute. The drug solution was injected using a 10 μl syringe, attached to the cannula with PE 20 polyethylene tubing. Flow was monitored by observing the movement of an air bubble over a calibrated length of the tubing. The cannula remained in place for 1 min after injection to minimize back flow of drug up the sheath. Each animal received only one microinjection. Non-specific effects were assessed by microinjecting butyrate, a breakdown product of dibutyryl cyclic nucleotides.

The following drugs were microinjected at sites in the CRF and PAG: dibutyryl cyclic 3′:5′-adenosine monophosphate (db cAMP, 10 μg, pH 4.5), dibutyryl cyclic 3′:5′-guanosine monophosphate (db cGMP, 20 μg, pH 4.5), butyrate (10 μg, pH 4.5). All drugs were obtained from Sigma Chemical Company (St. Louis, MO). Solutions of db cAMP and db cGMP were made up immediately before use.

Analgesiometric Testing

The antinociceptive activity of db cAMP and db cGMP was assessed using the tail flick (TF), paw pinch (PIN), and hot plate (HP) tests. Animals were tested on all three assays prior to and at fixed intervals following microinjection according to the following procedure. Antinociception was first assessed on the TF test, in which a high intensity beam of light was focused on the blackened tail. The time which elapsed between onset of the light and the reflex removal (flick) of the tail was defined as the tail flick latency. The average of three successive determinations was recorded.

Nociceptive threshold was next tested using the PIN test, by placing the hind paw between the jaws of a pincer (Stoelting Co., Chicago, IL); one jaw was flat and the other cone-shaped. The force exerted by the pincer was increased at a constant rate of 64 g/sec. The time at which the rat removed the paw, or struggled to do so, was recorded as the endpoint (Randall-Selitto test). The average of two successive determinations, one from each hindpaw, was taken as the latency for paw withdrawal.

Animals were then placed on a 55°C hot plate and the time required to lick a hind paw, jump, or squeal was defined as the hot plate latency. One determination was recorded. Any animal which failed to respond by 14 sec on the TF test, by 15.6 sec on the PIN test, or by 40 sec on the HP test, was removed from the test to minimize tissue damage, and assigned the maximum value.

Location of Injection Site

The location of the injection site in each rat was verified histologically and the position indicated on coronal sections drawn from the atlases of Pellegrino *et al.* [23] and Palkovits and Jacobowitz [22].

Statistical Analysis

Alterations in nociceptive threshold produced by db

cAMP, db cGMP, and butyrate were evaluated using one-way analysis of variance [11]. Comparisons of pre- versus post-drug mean response latencies were made using the Newman-Keuls test for multiple post hoc comparisons at individual time points [11].

RESULTS

Preliminary observations suggested that 10 μg db cAMP and 20 μg db cGMP were about equipotent in elevating TF latency following injection at sites in the CRF. These doses were also used in the present experiments to assess the antinociceptive potential of db cAMP and db cGMP injected at additional sites in the CRF and PAG. Butyrate was injected at CRF and PAG sites in other animals as a control for non-specific effects such as ionic strength, volume of injection, repetitive testing over the post-injection interval, and the effect of a major degradation product of the dibutyryl compounds.

Figure 1 shows the alterations in nociceptive threshold following microinjection of db cAMP, db cGMP and butyrate at CRF sites. Antinociception was considered to have occurred if (1) the response latency was elevated by at least two standard deviations above the mean pre-injection latency calculated for all animals and (2) this elevation occurred in at least two of three 10 min testing intervals immediately following injection. Pre-drug latencies±SD for the TF, PIN, and HP tests were 2.7±0.5 sec, 5.6±2.2 sec and 11.6±3.3 sec, respectively for the 74 animals used in this study. Figure 1 indicates that db cAMP and db cGMP both elevated the nociceptive threshold at the majority of CRF sites, as measured by all three tests. Butyrate produced antinociception only occasionally (2/10 rats). The magnitude, time course, and statistical significance of these effects are shown in Fig. 2. Microinjection of both db cAMP and db cGMP at CRF sites caused a significant elevation of TF, PIN and HP latencies ($p<0.05$ for each test). The effect of both agents was maximal within 10–20 min following microinjection in all three tests. The magnitude of the effect on the TF and HP tests had a tendency to decline during the 90 minutes testing period, but the elevated nociceptive threshold was never totally reversed. In some instances the antinociception lasted 3–6 hours (not shown). However, the elevated nociceptive threshold observed on the PIN test was totally reversed after 90 minutes. Injection of 10 μg butyrate at CRF sites did not cause a significant elevation in the TF or PIN latencies, but HP latency was significantly elevated (Fig. 2C). The duration of the effect of butyrate on HP latency, however, was much shorter than that of either db cAMP or db cGMP (Fig. 2C).

The dose-dependent nature of the antinociceptive effect of db cAMP was studied by microinjecting a lower dose of db cAMP (2 μg) at CRF sites (n=4, not illustrated). A significant elevation in the nociceptive threshold was observed at 10 min on the TF, PIN and HP tests when the magnitude of the effect was compared with pre-drug latencies. However, the magnitude of the effect of 2 μg db cAMP was not significantly different from that of 10 μg db cAMP (one-way ANOVA, $p<0.05$ for all tests), suggesting that these doses were at the upper limits of the dose-response curve. The duration of the antinociceptive effect on all tests was shorter after the 2 μg dose and reversal occurred within the 90 min test period.

Microinjection of 10 μg db cAMP at CRF sites did not cause any substantial disturbance in motor behavior. Some animals, however, appeared to be slightly ataxic and in some cases there appeared to be a reduction of movement in the open field. These animals nevertheless responded normally when placed on the hot plate. Similarly, db cGMP 20 μg generally did not produce substantial motor effects when microinjected at CRF sites, but slight ataxia was sometimes observed in those animals exhibiting analgesia. Aside from slight ataxia in some cases, microinjection of 10 μg butyrate at CRF sites had no apparent effect on motor performance.

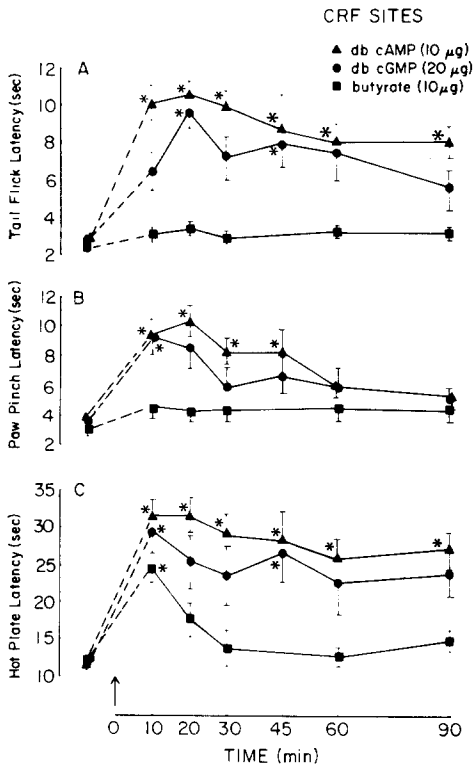

FIG. 2. Tail flick (A), paw pinch (B), and hot plate (C) latencies following microinjection (arrow) of db cAMP (n=21), db cGMP (n=14), and butyrate control (n=13) at the caudal reticular formation sites shown in Fig. 1. Each point represents the mean±SEM. *$p<0.05$, compared with pre-drug control value.

The antinociceptive efficacy of db cAMP and db cGMP was also tested following application of these agents in the PAG. Figure 3 shows the results of such experiments in which 10 μg db cAMP, 20 μg db cGMP, and 10 μg butyrate were microinjected at PAG sites. The criteria for antinociception in Fig. 3 are the same as those described for Fig. 1 (see above). Injection of db cAMP at most PAG sites induced antinociception as assessed by all three tests. By contrast, db cGMP was ineffective at most PAG sites and butyrate was effective at only one of nine PAG sites tested. The effect of db cAMP was statistically significant ($p<0.05$), was maximal within 10–20 min post-injection, and the majority of the animals regained their pre-drug test latencies within the 90 min testing period (Fig. 4). The marginal effect of db cGMP indicated in Fig. 3 was not statistically significant (Fig. 4).

Application of 10 μg db cAMP at sites in the PAG caused hyperactivity in about half the tested animals. In addition,

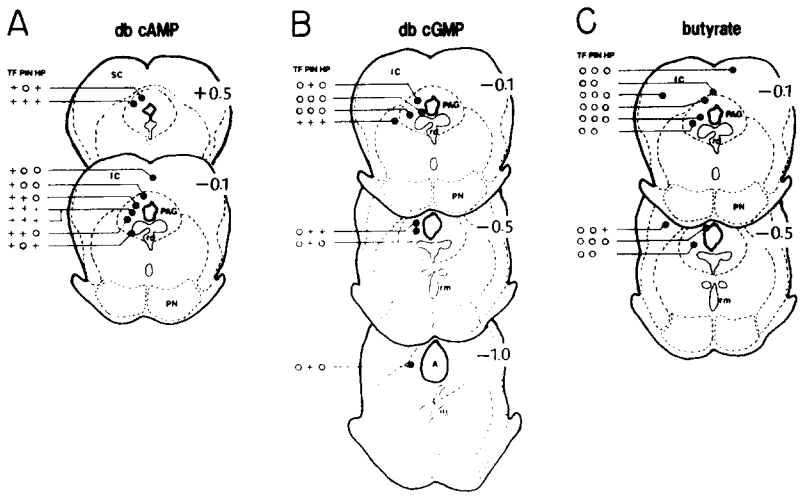

FIG. 3. Alteration in nociceptive threshold following microinjection of db cAMP (10 μg), db cGMP (20 μg), and butyrate (10 μg) at sites within and near the periaqueductal gray (PAG). Antinociception (+), or lack thereof (o), following administration at a given site was assessed on the tail flick (TF), paw pinch (PIN), and hot plate (HP) tests. See text for details. IC, inferior colliculus; SC, superior colliculus; PAG, periaqueductal gray; rd, nucleus raphe dorsalis; rm, nucleus raphe medianus; PN, pontine nuclei; III, nucleus of the third nerve. Each coronal section is numbered to indicate its distance (mm) rostral (+) or caudal (−) to the interaural line.

these animals often exhibited startle reactions and jumping both spontaneously and in response to innocuous stimulation. This hyperactivity was observed in those animals showing an elevation in their nociceptive threshold more often than in those that did not. When injected at PAG sites db cGMP 20 μg caused slight ataxia in some cases and two animals became hyperactive. Microinjection of 10 μg butyrate at PAG sites had no apparent motor effect.

DISCUSSION

We observed antinociception following microinjection of db cGMP at CRF sites, but not at sites in the PAG. Previous studies have shown that db cGMP causes analgesia following intracerebroventricular injection in rodents [1,28], but, as in the present study, is inactive at PAG sites [1]. It is possible, therefore, that the antinociception following intracerebroventricular injection of db cGMP reflects an action at CRF, but not at PAG sites. Although the PAG contains neuronal substrates for antinociception which are activated by opiates, the present results and those of Cohn *et al.* [1] indicate that these substrates cannot be activated by db cGMP.

The mechanism of the antinociception caused by administration of db cGMP at CRF sites is not known, but may involve modulation of transmission at cholinergic synapses in this area. Cyclic GMP has been proposed as a second messenger for cholinergic transmission [4, 21, 24]. CRF neurons are activated by cholinergic drugs [18] and microinjection of cholinergic agonists or cholinesterase inhibitors into the CRF causes an elevation in the nociceptive threshold (H. Proudfit, unpublished observations). Thus, the antinociception following microinjection of db cGMP at CRF sites may likewise reflect the activation of neurons in the CRF which receive cholinergic inputs.

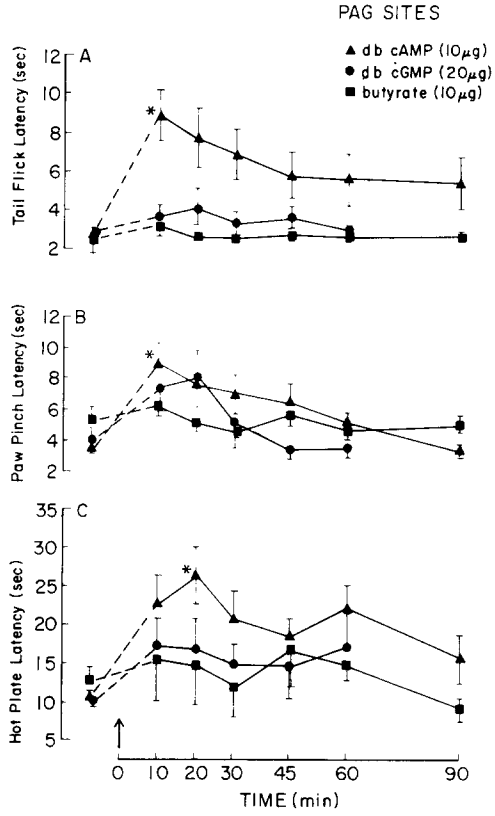

FIG. 4. Tail flick (A), paw pinch (B), and hot plate (C) latencies following microinjection (arrow) of db cAMP (n=9), db cGMP (n=7) and butyrate control (n=9) at the periaqueductal gray sites shown in Fig. 3. Each point represents the mean±SEM. *$p<0.05$, compared with pre-drug control value.

The induction of antinociception following microinjection of db cAMP at CRF and PAG sites is the first detailed report of an elevation in the nociceptive threshold produced by either systemic or local administration of a cyclic adenosine nucleotide [5,16]. Administration of 10 μg db cAMP at CRF sites produced an antinociceptive effect which lasted several hours. This long duration of action was probably the result of using a dose at the upper end of the dose-response curve; 2 μg db cAMP produced an effect of equal magnitude, but shorter duration (60–90 min). It is possible that the apparent antinociceptive effect of db cAMP at PAG sites is secondary to the motor disturbance observed. Gessa *et al.* [9] found that intracerebral injection of large doses of cyclic nucleotides (25 μg) in rat and cat produced severe motor disturbances such as convulsions and catatonia, sufficient to affect analgesiometric testing. However, the motor disturbances observed in this study were never as severe. Also, movement on the hot plate did not seem to be impaired despite the disturbance.

We also considered the possibility that the apparent elevation in the nociceptive threshold observed following introduction of db cAMP at CRF sites was secondary to an alteration in cardiovascular function, since descending projections from the CRF innervate the lateral horn of the spinal cord [3]. In addition, administration of db cAMP into the lateral ventricle of cat caused elevation in blood pressure [6]. Such a secondary effect seems unlikely however, since microinjection of 10 μg db cAMP (and 20 μg db cGMP) at CRF sites produced a minimal effect (about 10 mm Hg elevation) on blood pressure (R. Levy, unpublished observations).

The capacity of both db cAMP and of morphine to cause an elevation in the nociceptive threshold when microinjected into the CRF and PAG suggests the possibility that these agents may activate the same neuronal pathway. Several lines of evidence support the involvement of cAMP in the mediation of opiate analgesia [25]. However, opiates decrease adenylate cyclase activity in neuroblastoma × glioma cells [13] and inhibit the prostaglandin-sensitive adenylate cyclase in rat brain homogenates [2]. In addition, prior administration of cAMP into the rat lateral ventricle decreased the antinociceptive action of morphine [11]. These observations suggest that administration of db cAMP should lower nociceptive threshold. However, elevation in such threshold (analgesia) followed the administration of db cAMP at CRF and PAG sites, which suggests that the action of db cAMP at these sites is not related to the mechanism of morphine analgesia. Furthermore, the failure of iontophoretically applied db cAMP, cAMP or phosphodiesterase inhibitors to alter the response of dorsal horn cells to noxious peripheral stimulation, or to alter the depression of these cells by morphine, suggests that cAMP is not involved in the action of morphine at the spinal level as well [7].

The antinociception observed following microinjection of db cAMP at CRF and PAG sites may reflect actions at monoaminergic or cholinergic synapses. It is possible, for example, that db cAMP augments the activity of serotonergic neurons, located in the raphe magnus of the CRF, which have been implicated in the control of nociceptive threshold via their descending projections to the spinal cord [8, 19, 20, 29]. Tagliamonte *et al.* [27] have shown that db cAMP augments the rate of serotonin synthesis in rat brain following injection of db cAMP into the lateral ventricle. Alternatively, db cAMP may cause analgesia at CRF sites through its alleged role as second messenger for noradrenergic transmission [4, 21, 24]. Noradrenergic terminals of unknown origin are found in the immediate vicinity of the raphe magnus [10,14] and this noradrenergic input seems to decrease nociceptive threshold since its blockade by local injection of noradrenergic antagonists caused analgesia [10]. However, microinjection of db cAMP in this area (present studies) also caused analgesia, not the reduction in nociceptive threshold expected following activation of these noradrenergic synapses. Thus, the present results with db cAMP at CRF sites probably do not reflect an action at noradrenergic synapses.

cAMP has been reported to augment the release of acetylcholine at the neuromuscular junction [4,24]. The analgesia following microinjection of db cAMP at CRF sites may likewise reflect facilitation of ACh release from cholinergic terminals in the CRF (see above). Similarly, the analgesia produced by microinjection of db cAMP at sites in the PAG may result from modulation of cholinergic [22,26] and/or noradrenergic [14,22] inputs terminating in this area. Implicit in these proposed mechanisms of action is the assumption that the local application of these agents mimics the actions of endogenous cyclic nucleotides at synapses where cyclic nucleotides act as second messengers or modulators of synaptic activity. The observed antinociception following injection of db cAMP and db cGMP may, however, represent non-specific actions at synapses which do not utilize cyclic nucleotides. However, recent studies in this laboratory have shown that phosphodiesterase inhibitors will also elevate the nociceptive threshold when microinjected into brainstem sites [15].

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