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ESTIMATION OF THE AFFINITY OF NALOXONE AT SUPRASPINAL AND SPINAL OPIOID RECEPTORS IN VIVO: STUDIES WITH RECEPTOR SELECTIVE AGONISTS

Julius S. Heyman, [a]Randy J. Koslo, [b]Henry I. Mosberg
[c]Ronald J. Tallarida and Frank Porreca

Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ 85724, [a]Bristol-Myers Products, Hillside, NJ 07207, [b]College of Pharmacy, University of Michigan, Ann Arbor, MI 48109 and [c]Department of Pharmacology, Temple University Medical School, Philadelphia, PA 19102

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Summary

The apparent affinity of naloxone at cerebral and spinal sites was estimated using selective mu [D-Ala^2, Gly-ol^5]-enkephalin (DAGO) and delta [D-Pen^2, D-Pen^5]enkephalin] (DPDPE) opioid agonists in the mouse warm water tail-withdrawal test in vivo; the mu agonist morphine was employed as a reference compound. The approach was to determine the naloxone pA_2 using a time-dependent method with both agonist and antagonist given intracerebroventricularly (i.c.v.) or intrathecally (i.th.); naloxone was always given 5 min before the agonist. Complete time-response curves were determined for each agonist at each site in the absence, and in the presence, of a single, fixed i.c.v. or i.th. dose of naloxone. From these i.c.v. or i.th. pairs of time-response curves, pairs of dose-response lines were constructed at various times; these lines showed decreasing displacement with time, indicative of the disappearance of naloxone. The graph of log (dose ratio - 1) vs. time was linear with negative slope, in agreement with the time-dependent form of the equation for competitive antagonism. From this plot, the apparent pA_2 and naloxone half-life was calculated at each site and against each agonist. The affinity of naloxone was not significantly different when compared between agonists after i.c.v. administration. A small difference was seen between the affinity of i.th. naloxone against DPDPE and DAGO; the i.th. naloxone pA_2 against morphine, however, was not different than that for DPDPE and DAGO. The naloxone half-life varied between 6.6 and 16.9 min, values close to those previously reported for this compound. These results suggest that the agonists studied may produce their i.c.v. analgesic effects at the same receptor type or that alternatively, the naloxone pA_2 may be fortuitously similar for mu and delta receptors in vivo. Additionally, while the affinity of naloxone appears different for the receptors activated by i.th. DAGO and DPDPE, further work may be necessary before firm conclusions regarding the nature of the spinal analgesic receptor(s) can be drawn.

Recent work in this laboratory (1,2) and that of others (3,4) has attempted to investigate the role of central delta opioid receptors in various opioid

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mediated effects such as analgesia, slowing of gastrointestinal propulsion, production of respiratory depression and induction of dependence. These studies have employed the most selective agonists presently available for mu (DAGO)(5) and delta (DPDPE)(6) opioid receptors. The development of selective agonists such as DPDPE has finally begun to allow some insights as to the physiological function of these receptors. Questions regarding the role of delta receptors have remained, however, since even selective agonists such as DPDPE may produce their opioid effects at other (mu?) types of receptors.

A traditional approach in the differentiation of receptors has been the determination of antagonist affinity against various agonists. This approach was extensively and successfully employed in opioid research by Takemori and his colleagues both in vitro (7) and in vivo (8,9). The method involves determination of the agonist dose-response relationship in the absence and in the presence of several doses of antagonist. The resulting dose ratios are then used in the construction of a Schild plot (10) from which the antagonist affinity is determined (see 11, for review). Application of this method in vivo, however, presents some special problems since the administered doses of agonist and antagonist are used in the calculation with the assumption that these doses are proportional to the tissue concentration. Further, the tissue concentration is assumed to be maximal at the time of peak effect and the measurement of effect must be made at this time. As there may be a dose-related time to peak effect, it is often difficult, if not impossible, to make the measurement of effect at the appropriate time for each dose. These factors are among those which make difficult the interpretation of pA_2 values obtained with studies in vivo. Additionally, a more practical difficulty arises from the quantity of agonist needed to determine the dose-ratios necessary for construction of the Schild plot. While this concern is not normally important, the availability of some peptides, such as DPDPE, has been limited.

The present investigation attempts to determine whether naloxone has similar affinity at cerebral and spinal opioid receptors activated by the delta agonist, DPDPE, or the mu agonists morphine and DAGO. A variation of the traditional pA_2 method has been employed. This method includes time as a variable and thus obviates many of the concerns of pA_2 studies in vivo, such as the making of measurements at the time of peak effect and the assumption of equilibrium conditions. This method has been previously derived (12) and validated (13). Additionally, the present study administered both the agonist and the antagonist into the central nervous system. Administration of compounds by these routes improves precision by delivering compounds close to their loci of action thereby permitting a dose unit more closely approximating tissue concentration as well as more predictable pharmacokinetics. We now report that the naloxone pA_2 against mu (DAGO, morphine) and delta (DPDPE) agonists is not significantly different after i.c.v. administration of these compounds. A small difference in the naloxone pA_2 was seen against DAGO and DPDPE after i.th. administration. Additionally, the half-life of naloxone (and 95% confidence limits) was determined as a by-product of the calculations, and this value was found to be similar to those previously reported.

Methods

Mouse warm-water tail-withdrawal test

Experiments were made using male, ICR mice (25-30 g). The animals were

housed in groups of 5 in a temperature controlled room with a 12 hr light-dark cycle (lights on at 7.00 AM). Food and water were continuously available. On the day of the experiment, each mouse was tested for a control response to a noxious stimulus. The tail was immersed in warm water (55°C) and the time to a rapid flick determined. Groups of mice then received an i.c.v. or i.th. injection of saline or naloxone, followed after 5 min by a dose of i.c.v. (in the same ventricle) or i.th. agonist. Testing took place after a further 5, 10, 20, 40, 60, 80, 100, and 120 min. Each response latency was then compared to the individual control latency and percent analgesia for each mouse expressed as:

% analgesia = 100 x (test latency - control latency)/(15 - control latency), with 15 sec chosen as the maximum time for tail-withdrawal. Animals not removing their tails within 15 sec were scored as having 100% analgesia.

Theory

The time-dependent form of the equation for competitive antagonism was employed as previously derived by Tallarida and associates (12). Briefly, the dissociation constant (K_B) of a competitive antagonist of concentration B is computed by the equation of Arunlakshana and Schild (14):

$\log (A'/A - 1) = \log (B) - \log K_B$, where A' and A are equieffective agonist concentrations in the presence and in the absence of the antagonist. Although the determination of antagonist affinity can be made directly from the above equation, a standard approach is to determine the dose ratio in the presence of several concentrations of antagonist and to plot log (dose ratio - 1) vs. log B (the Schild plot). This plot is theoretically linear with slope equal to 1 and intercept equal to $-\log K_B$ (ordinate), or pA_2 (abscissa)(14). A modification of the above equation which considers time assumes that both agonist and antagonist concentration decrease exponentially after reaching maximum concentration has been achieved. Thus, $A = A_0e^{-at}$, and $B = B_0e^{-bt}$, where A_0 and B_0 are the maximum concentrations (assumed proportional to the administered concentrations), a and b are the rate constants for the disappearance of A and B, respectively and t is the time after peak effect. Thus, t = 0 corresponds to the time of peak effect. From these, the time dependent form of the Arunlakshana and Schild equation is:

$\log (A'/A - 1) = \log B_0 - b\log (e)t - \log K_B$. If $\log (A'/A - 1)$ is plotted against time, a straight line results of slope = $-b\log (e)$ and intercept = $(\log B - \log K_B)$. As $pA_2 = -\log K_B$, then pA_2 = intercept $-\log B_0$. Additionally, the rate constant, b, for the disappearance of the antagonist, and resulting half-life, follows. It should be pointed out that by using the time-dependent method one does not need to make assumptions regarding the time of peak effect of the agonist and the agonist plus antagonist. This consideration increases the suitability of this approach for study in vivo.

Statistics

All dose-response lines were constructed by standard regression techniques as described by Tallarida and Murray (15). Dose-ratios were determined by

establishing the best parallel regression lines in the absence and in the presence of all naloxone doses. The 95% confidence limits for the pA_2 value of each agonist and route were estimated from the error of the intercept on the ordinate; thus, the 95% C.L. was "t" times the standard error of the intercept, where "t" represents the area under the t-distribution curve based on the size of the sample. The 95% C.L. of the naloxone half-life was similarly determined from the confidence limits of the slope of the regression line obtained when plotting log (dose ratio - 1) vs. time.

Results

Time-response curves for morphine, DAGO and DPDPE in the absence (top row) and in the presence (bottom row) of a single i.c.v. dose of naloxone (0.5, 0.5 and 1 ug, respectively) are shown in Fig. 1 and for a single i.th. dose of naloxone (1, 0.5 and 0.1 ug) are shown in Fig. 2. The agonists showed a duration of action after i.c.v. or i.th. administration ranging between 80 and 100 min. From these paired curves, the response in the absence, and in the presence, of naloxone was plotted at each dose of agonist at various times. An example of such a plot for i.c.v. DPDPE is shown at 20, 25, 35 and 40 min in Fig. 3. These plots for other agonists and routes were similar, showing a decrease in dose-ratio with time, indicative of the disappearance of naloxone. The log (dose ratio - 1) obtained from plots such as those in Fig. 3 are shown for each agonist and route in Fig. 4. Each of the lines showed excellent linear correlation, strongly supporting the validity of the theoretical assumptions. Note that the slopes of these lines (Fig. 4) do not reflect whether antagonism is competitive (as shown by a slope of unity in the traditional Schild plot) but are the result of the kinetics of naloxone disappearance from the biophase. From the plots in Fig. 4, the pA_2 (and corresponding 95% C.L.'s) were obtained from each agonist and route (Table I). Additionally, the naloxone half-life (and 95% C.L.'s) against each agonist and route (given by the slope of the lines in Fig. 4) is shown in Table I. Finally, the peak A_{50} (and 95% C.L.'s) obtained from the time-response curves in the absence of naloxone (Fig. 1,2) are also shown in Table I.

The apparent naloxone pA_2 values for i.c.v. morphine, DAGO and DPDPE were 10.39 (10.29 - 10.96), 9.99 (9.28 - 10.69) and 10.63 (10.14 - 10.64), respectively. Similarly, after i.th. administration of morphine, DAGO and DPDPE, the pA_2 values were 10.16 (9.91 - 10.43), 9.74 (9.5 - 9.98) and 10.15 (10.12 - 10.18), respectively. The overlapping 95% C.L.'s after i.c.v. administration indicate a lack of significant difference between these values when compared between agonists. The values seen after i.th. administration indicate no overlap between the naloxone pA_2 against DAGO and DPDPE, although the value against morphine was not different from either of the other agonists. The half-life of naloxone was found to range approximately between 9 and 17 min (see Table I), values that compare well with those previously reported by the same, and other techniques (13,16). An exception was the briefer naloxone half-life found with i.c.v. morphine (6.6 min). Additionally, a comparison of peak A_{50} values of each compound after i.c.v. and i.th. administration shows that morphine and DAGO are more potent i.c.v. (3.6 and 8.3 fold, respectively) while DPDPE is essentially equipotent by the two routes.

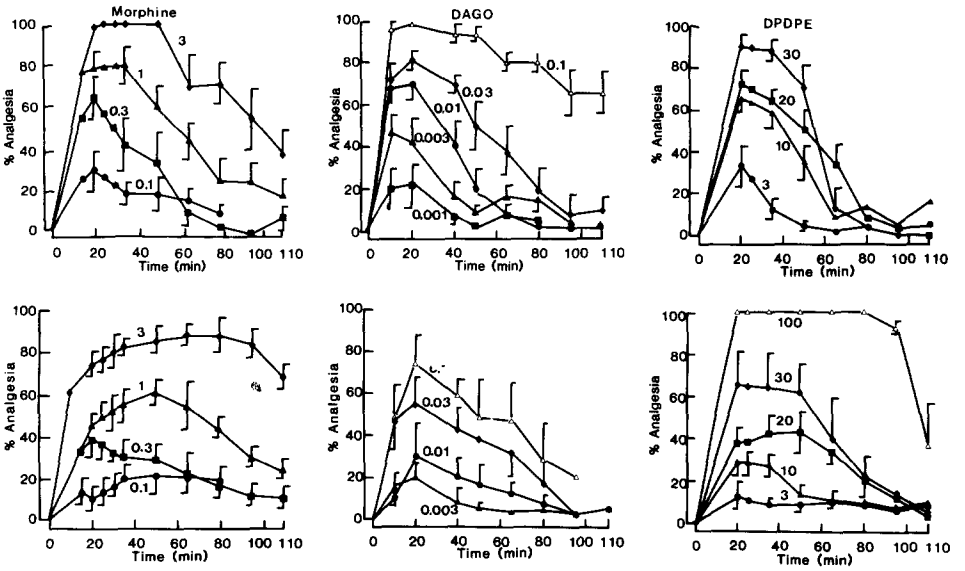

Figure 1. Time-response curves for i.c.v. morphine, DAGO and DPDPE analgesia in the absence (top row) and in the presence (bottom row) of a single, i.c.v. dose of naloxone (0.5, 0.5 and 1 µg, respectively) given 5 min prior to testing. Testing took place 20 min after the agonist. Dose given is shown by the value next to each curve (µg/mouse).

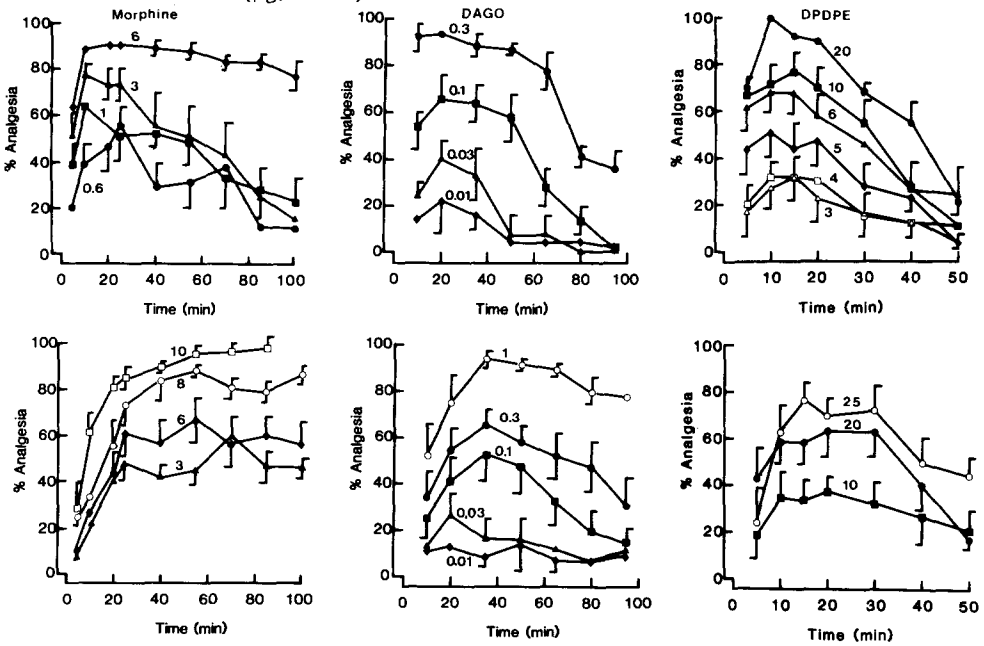

Figure 2. Time-response curves for i.th. morphine, DAGO and DPDPE analgesia in the absence (top row) and in the presence (bottom row) of a single, i.th. dose of naloxone (1, 0.5 and 0.1 µg, respectively) given 5 min prior to testing Testing took place 20 min after the agonist (+10 min for DPDPE). Dose given is shown by the value next to each curve (µg/mouse).

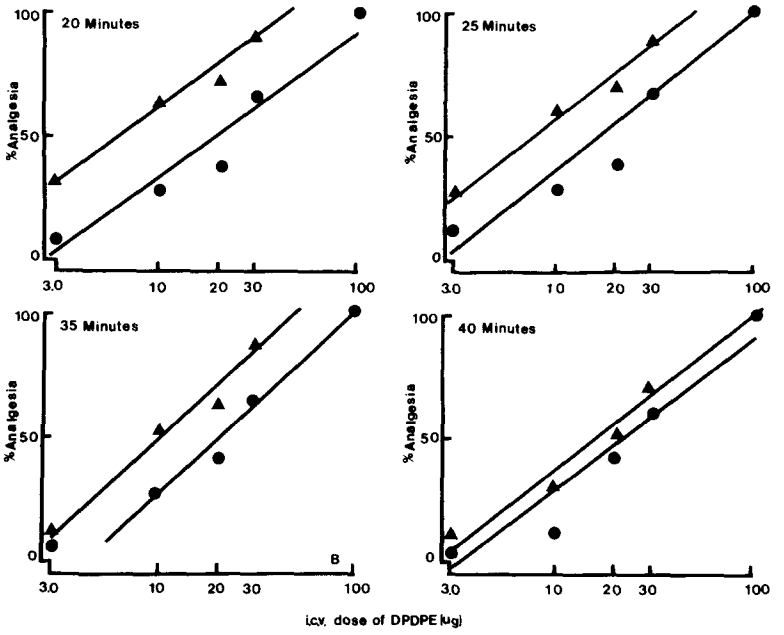

Figure 3. Dose-response lines at various times determined from time-response data for i.c.v. DPDPE/naloxone in the mouse tail-withdrawal test.

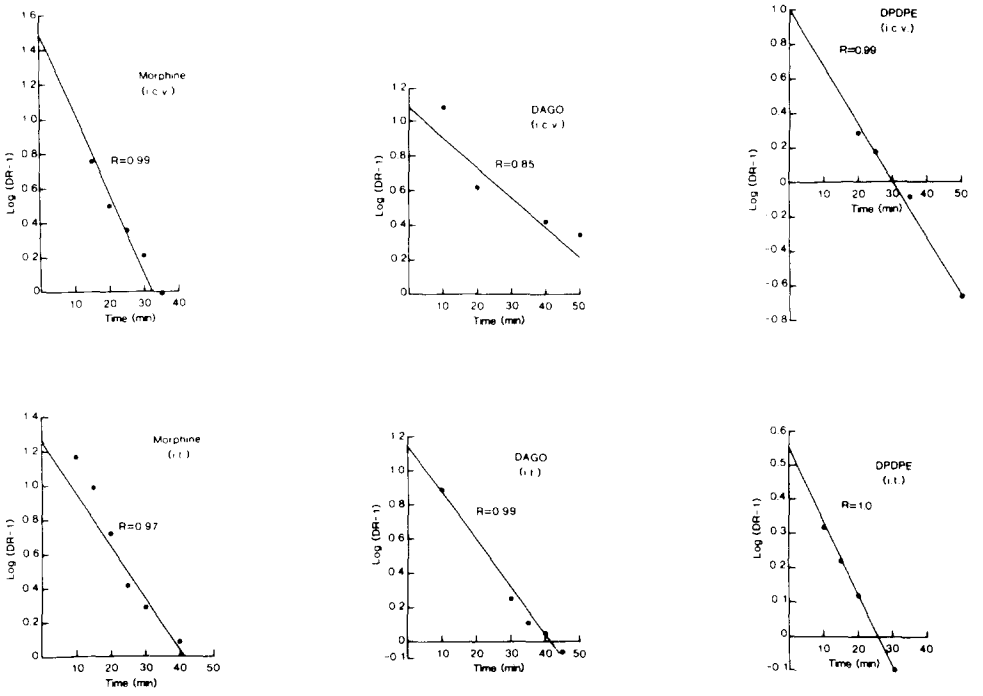

Figure 4. Plots of log (dose-ratio - 1) vs. time for i.c.v. (left panel) and i.th. (right panel) morphine, DAGO and DPDPE in the mouse tail-withdrawal test. See text for details.

TABLE I

Time-dependent apparent pA_2 for naloxone against DAGO, morphine and DPDPE after i.c.v. or i.th. administration. Naloxone was given at - 5 min, agonists at t = 0, and testing occurred at + 20 min for all compounds except i.th. DPDPE (+ 10 min) in the mouse warm water tail-immersion test.

Agonist	Route	pA_2 (95% C.L.)	$t_{1/2}$ (95% C.L.)(min)	A_{50} (ug, peak)
Morphine	i.c.v.	10.39(10.14-10.64)	6.6 (5.6-7.9)	0.21(0.075-0.61)
DAGO	i.c.v.	9.99(9.28-10.69)	16.99(7.9-26.1)	0.005(0.003-0.007)
DPDPE	i.c.v.	10.63(10.29-10.96)	9.1 (6.4-16.1)	6.11(3.76-9.93)
Morphine	i.th.	10.16(9.91-10.43)	10.2 (7.4-16.3)	0.76(0.25-2.31)
DAGO	i.th.	9.74(9.50-9.98)	10.89(8.7-14.7)	0.042(0.03-0.06)
DPDPE	i.th.	10.15(10.12-10.18)	14.22(13.4-15)	5.2(4.08-6.67)

Discussion

The present study has determined the apparent pA_2 of naloxone against agonists selective for delta (DPDPE) and mu (DAGO) receptors, with morphine employed as a reference agonist. The approach involved the determination of this affinity constant at cerebral and spinal sites using a time-dependent method. Critically, both the agonist and the antagonist were given either into the brain or into the spinal subarachnoid space. In particular, administration of the antagonist close to its site of action increases the precision of the determination of affinity. The values obtained, however, are not directly comparable to studies using different routes of administration. It has been well established that values of pA_2 in vivo, are dependent on the route of compound administration (11). It follows, therefore, that the values of the naloxone pA_2 reported after i.c.v. administration are not directly comparable to the values reported after i.th. naloxone. This conclusion is supported by the different time-response curves (Fig. 1, 2).

The time-dependent method was originally derived by Tallarida and colleagues (12) as a check on the standard method of Schild (10). The results were found to agree well with the traditional method in that study and in a subsequent validation of the method when naloxone was given by a central (i.c.v.) route (13). Our results indicate a lack of significant difference between the affinity of naloxone for the cerebral receptors activated by DPDPE and those activated by DAGO and morphine. A difference in naloxone affinity against these receptor selective agonists would be interpreted as suggesting that the agonists were acting at different receptor populations. Similar affinity suggests either that the agonists are activating the same receptor population, or that there is a fortuitous similarity in naloxone affinity at two different receptor populations. The present work cannot distinguish between these possibilities. It seems possible that the affinity of the

agonists for each receptor may be close enough that pA_2 determinations in vivo are unable to discriminate between them. Alternatively, the similarity in values argues that the i.c.v. analgesic receptor activated by DPDPE, morphine and DAGO are the same. It should be noted, however, that significant evidence indicates that DPDPE acts at receptors different from those acted upon by i.c.v. morphine. For example, Porreca et al. (1) have reported that while i.c.v. morphine and DAGO produce both analgesia and inhibition of gastrointestinal transit, i.c.v. administration of DPDPE results only in analgesia. In consequence, the lack of difference between the naloxone pA_2 against morphine, DAGO and DPDPE is difficult to explain.

An evaluation of the naloxone pA_2 values after spinal administration shows that the confidence limits of this antagonist against morphine overlap with those of DAGO and DPDPE. The affinity of naloxone appears to differ, however, between i.th. DPDPE and i.th. DAGO. The magnitude of this difference, however, is very small, and in our view, probably does not represent a meaningful difference. Thus, DPDPE and DAGO, the most selective agonists for the delta and mu receptors, respectively, may be acting at different spinal receptors in the production of analgesia. Caution should be exercised, however, as the confidence limits of the naloxone pA_2 against i.th. DPDPE are unusually small, due in part to the excellent fit of the log (dose ratio - 1) vs. time plot. These unusually tight confidence limits may lead to incorrect conclusions. It should be noted, that the question of opioid receptor involvement in antinociception has been previously addressed using other approaches, especially at the level of the spinal cord. In particular, Yaksh and colleagues have addressed this issue in a series of work which has supported the separate involvement of spinal and supraspinal mu and delta receptors in antinociception. Tung and Yaksh (16) demonstrated different pA_2 values for i.p. naloxone against i.th. ethylketocyclazocine and [D-Ala^2, D-Leu^5]enkephalin (DADLE). Furthermore, in rats tolerant to morphine, that study showed no change in the i.th. DADLE dose-response curve. This conclusion was supported by the work of Tseng (17) who demonstrated only partial cross-tolerance between morphine and DADLE spinal anticociception in the rat. Additional work by Yaksh in the primate (18) using many approaches also concluded the involvement of both mu and delta receptors at the spinal level, a conclusion also supported in later work in the rat (19) which also suggested the involvement of spinal kappa receptors in visceral chemical, but not thermal, stimuli. Finally, very recent work (20) has demonstrated the involvement of supraspinal delta opioid receptors, in addition to mu receptors, in antinociception in tests utilizing heat as the nociceptive stimulus. Jensen and Yaksh (20) have reported that mu (morphine and sufentanil) and delta (DADLE, [D-Ser^2, Leu^5, Thr^6]enkephalin) agonists were effective in the tail flick test when given directly into the periaqueductal gray. In contrast, only the delta agonists were effective in this test when the compounds were given into the medullary reticular formation. Thus, the results of various approaches must be integrated before a final conclusion regarding the nature of the spinal analgesic receptor acted upon by DPDPE and DAGO or morphine can be drawn; the small difference in the naloxone pA_2 against DAGO and DPDPE can be evaluated only as one piece of evidence.

This study provides an attempt to determine the nature of the analgesic effect of a highly selective agonist at the delta receptor. Additional information regarding the time-course for these agonists at different routes is also provided. It is interesting that the doses of naloxone necessary to provide intermediate levels of antagonism varied for each compound and for each route, with i.th. DPDPE being the most sensitive to antagonism (only 0.1 ug naloxone necessary). The reasons for these differences in naloxone dose are unclear, but may relate to the pharmacokinetics of the compounds at the

spinal and supraspinal levels. Finally, the half-life of naloxone was relatively consistent with results reported by other methods (approximately 13 - 20 min) (13,21). The naloxone half-life against i.c.v. morphine, however, was briefer than expected; the reasons for this are unclear.

In summary, the present study demonstrates that within the limits of our methodology, the affinity of naloxone for mu and delta receptors as revealed by selective agonists (DAGO, DPDPE) is very similar. The results obtained using the i.th. route indicate the possibility of a small difference between the affinity of naloxone for the receptors activated by DPDPE and DAGO. The interpretation of this result is made difficult, however, due to (a) the small confidence limits of the i.th. naloxone-DPDPE pA_2 and, (b) the small difference in the absolute magnitude of the two affinity constants. Further work using selective antagonists is required to establish whether these agonists produce their analgesic effects at the same or separate receptors. Additionally, pharmacokinetic data have been established for i.c.v. and i.th. administration of these agonists, and morphine, in the absence and in the presence of naloxone. Such information may be useful in the design of future experimental protocols with these agonists.

Acknowledgments

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