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MODIFICATION OF OPIOID LIGAND BINDING IN THE CENTRAL AND THE PERIPHERAL NERVOUS SYSTEM BY DIFFERENT BUFFERS

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SUMMARY : The modification of binding parameters (equilibrium dissociation constant and binding capacity) of three opioid ligands (DADLE, Etorphine and EKC) on bovine adrenal medulla and rat brain membranes have been examined in three buffer systems : Tris-HCl 50 mM, Hepes-NaOH 10 mM and Tes-KOH 10 mM. Major differences of these parameters have been found : Hepes-NaOH provoked a diminution of the apparent number of binding sites, while a concomitant diminution of the K_D and Bmax was observed in Tes-KOH buffer. Substitution of counterions in these two buffers produced further changes of binding characteristics : in Hepes buffer we have observed an abolition of ^3H DADLE binding, an enhancement of ^3H EKC binding and no modification of ^3H etorphine binding characteristics. On the contrary an abolition of the specific binding of all three ligands in Tes buffer was found in the bovine adrenal medulla while minor changes were observed in rat brain. It is concluded that, inspite same disadvantages (substitution for bivalent cations and temperature dependence), Tris-HCl is the buffer of choice for the analysis of opioid binding site interactions.

Tris-HCl (1,2) and Hepes (3,4) are the most commonly employed buffer in studies in which the interaction of opioid ligands with their binding sites is investigated. Nevertheless the use of the former buffer is rather unconfortable in studies in which the effect of ions is assayed or whether temperature is a parameter to take into consideration (for example thermodynamic studies). Indeed, Tris-HCl can substitute for bivalent cations and its pH is temperature dependent.

In the present study we have investigated whether the replacement of Tris-HCl with some recently developped zwitterionic buffers could modify the binding parameters of opioid ligands to their sites. As the bovine adrenal medulla, used in our laboratory to investigate opiate-binding sites interactions, is not a tissue commonly used in opioid studies, a compa-

Abbreviations

DADLE : D-Ala^2, D-Leu^5 Enkephaline
EKC : Ethylketocyclazocine
Hepes : N-(2-Hydroxyethyl) piperazine-N'-2-Ethanesulfonic acid
Tes : N-Tris (hydroxymethyl) methyl 2-aminoethane-sulfonic acid

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rison has been equally made with results obtained in rat central nervous system.

METHODS AND MATERIALS

1. Buffers : In addition to Tris-HCl 50 mM (pH 7.4), a buffer previously used in our laboratory for opioid binding site determination, two zwitterionic buffers have been used : Hepes-NaOH (10 mM, pH 7.4) and Tes-KOH (10 mM pH 7.4). In some cases, (see Results) these buffers were treated with ion-exchange resines (Chelex 100 followed by BioRad AG501x8 (D) in order to exchange Na^+ or K^+ counterions respectively (5). In the case of Hepes-NaOH buffer this treatment provoked a shift of the pH towards basic values compensated by the addition of HCl. In the case of Tes-KOH buffer a shift towards acid pH was observed. In this latter case it was necessary to readjust the pH with small amounts of NaOH. In neither case this ion-exchange resin treatment did modify the capacity of the buffers, as assayed by their titration curves with NaOH or HCl.

2. Opioid ligand binding : Bovine adrenal were obtained from a local slaughterhouse. They were kept on ice and transfered to the laboratory within one hour from the death of the animal. Medulla was rapidly separated from cortex, and crude membranes were prepared by differential centrifugation between 1500 and 30 000 x g, in Tris-HCl buffer. After two additional washes, and a 30 min preincubation at 37°C (in order to dissociate any endogenous opioid peptide) in Tris-HCl buffer, membranes were resuspended in each buffer to be tested, washed twice and finally resuspended to give a concentration between 1 and 2 mg protein/ml as determined by the method of Lowry et al with BSA as a standard (6). Rat brain membranes were prepared with a similar technique. Adult male Sprague-Dawley rats (180-220g) were decapitated and whole brain, minus cerebellum, was used for membrane preparation as described above. Opioid binding was performed in a final volume of 1.0 ml, containing 0.5 ml membrane suspension, 0.1 ml radioligand and 0.4 ml assay buffer. Incubation was performed at 37°C for 30 min. Separation of bound and free radioactivity was made by rapid filtration through Whatman GF/B glass-fiber dishes presoacked with assay buffer (7,8). Non specific binding was determined in the presence of 10 µM levorphanol. Analysis of binding isotherms was performed by linear regression methods from the Scatchard plots of the data (9) or by mathematical modeling using the "LIGAND" program (10).

3. Materials : ^{3}H DADLE (specific activity 45 Ci/mmol) and ^{3}H EKC (specific activity 15 Ci/mmol) were purchased from New England Nuclear corporation. ^{3}H Etorphine was purchased from Amersham. Levorphanol was a gift from Hoffmann-LaRoche. Ion exchange resines (Chelex 100 and AG501 x 8 (D) were from BioRad Laboratories (Richmond CA). All other chemicals were purchased from Merck (Darmstadt FRG).

RESULTS

Table 1 presents the modification of binding parameters of opioid ligands on bovine adrenomedullary membranes in different buffer systems. Major modifications could be observed : as compared to Tris-HCl, experiments carried out in Hepes-NaOH buffer produced minor modifications of the affinity of opioid ligands but a significant decrease of the apparent number of binding sites. On the contrary, incubation in Tes-KOH buffer produced a diminution of both the affinity and the binding capacity of opioid ligands as compared to Tris-HCl buffer, with the exception of ^{3}H etorphine, in which case a minor enhancement of the affinity of the radioligand was found. It

Table 1 : Modification of equilibrium binding parameters of opioid binding on bovine adrenomedullary membranes upon incubation in different buffers

Ligands	Tris-HCl		Tes-KOH		Hepes-NaOH		Tes-NaOH*		Hepes-HCl*	
	K_D	Bmax	K_D	Bmax	K_D	Bmax	K_D	Bmax	K_D	Bmax
^{3}H DADLE	0.90	4	2.25	3	1.57	2	ND		ND	
^{3}H Etorphine	0.41	111	0.25	56	0.46	40	ND		0.60	48
^{3}H EKC	0.20	11	ND		0.18	10	ND		0.12	31
	3.20	98	4	70	3.70	89			3.80	148

: Tris-HCl (50 mM), Tes-KOH and Hepes-NaOH (10 mM). The pH of all buffers was adjusted to 7.4. No modification of binding parameters were observed when 50 mM Tris-HCl was replaced by a 10 mM buffer. *:Zwitterionic buffers were treated by ion-exchange resines prior to incubation as described under "Methods and Materials".
K_D : nM; Bmax : fmoles/mg protein; ND : not detectable.

is of interest to note that incubation in Tes-KOH buffer produced a complete abolition of ^{3}H EKC high affinity binding on membranes.

Previous results from this (11,12) and other laboratories (13,14) indicated that monovalent ions could influence the affinity and/or the binding capacity of opioid ligands for their sites. We have therefore examined whether the observed differences in binding parameters were due to the buffer itself or to the counterions used. Results are equally presented in table 1.

Substitution of Na^+ for K^+ in Tes-buffer produced a complete abolition of binding in all three ligand systems. On the contrary, substitution of Na^+ by H^+ in Hepes buffer produced an abolition of ^{3}H DADLE binding, no modification of ^{3}H etorphine binding parameters, and an enhancement of the number of ^{3}H EKC identified high and low affinity sites.

Adrenal medulla is not a tissue currently used for opioid-binding site interactions. In order to investigate whether buffer substitution influences opioid binding in other tissues, a similar series of experiments have been performed in the rat central nervous system. This latter tissue has been abondantly used for the assay of opioid-binding site interactions. Our results are presented in table 2. A significant decrease of equilibrium binding parameters was obtained by the substitution of Tris-HCl by Hepes buffer. This decrease was comparable in the two tissues for DADLE and etorphine binding while it was not detected during ^{3}H EKC high affinity binding in the bovine adrenal medulla. Opioid binding performed in Tes-KOH showed smaller variations than in the bovine adrenal medulla, with the exception of a significant decrease of ^{3}H DADLE identified sites. It is of interest to note that a high affinity site was detected in rat brain only during

Table 2 : Modification of equilibrium opioid binding parameters on rat brain membranes upon substitution of Tris-HCl by zwitterionic buffers

Ligands	Tris-HCl		Tes-KOH		Hepes-NaOH		Tes-NaOH*		Hepes-HCl*	
	K_D	Bmax	K_D	Bmax	K_D	Bmax	K_D	Bmax	K_D	Bmax
^{3}H DADLE	0.26	52	0.06	17	2.40	36	0.42	45	1.37	28
^{3}H Etorphine	0.26	186	0.24	164	0.86	64	0.27	129	0.19	106
^{3}H EKC	0.15	30					0.03	25		
	0.29	59	0.29	60	0.46	50			0.26	43
			1.20	143	5.02	303	0.65	86	1.46	158

Binding conditions and symbols are explained in the legend of table 1.

^{3}H EKC binding in Tris-HCl buffer. Curve fitting using the statistical methods developped by Munson and Rodbard (10) showed a significantly better fitting using the two sites over the one site model. On the contrary, during ^{3}H EKC binding in Tes-KOH or Hepes-NaOH buffer a low affinity, high capacity binding site could be detected. Similar, although less pronounced, results were obtained in rat brain as compared to the bovine adrenal medulla, (^{3}H DADLE and ^{3}H etorphine binding) after treatment of buffers with ion exchange resines. It is of interest to note that treatment of Tes buffer restored the high affinity ^{3}H EKC binding obtained during Tris-HCl.

DISCUSSION

As indicated above (see Introduction) Tris-HCl buffer, commonly used in ligand-binding site interactions presents some minor disadvantages. It can substitute for bivalent cations and its pH is temperature-dependent. In order to overcome this second point, Hepes buffer has been previously employed in thermodynamic and kinetic studies in which temperature was a critical factor (15,16). Recently Barnard and Demoliou-Masson have proposed the use of Tes-KOH buffer to substitute for Tris-HCl (13). This latter buffer has a pK of 7.50 and presents non interaction with divalent cations. We have therefore examined whether either of these zwitterionic buffers could substitute for Tris-HCl for the interaction of opiates with their binding sites in the bovine adrenal medulla.

The results of the present study indicate that major differences of binding capacity and/or affinity can be detected in the three buffer systems. In general Hepes-NaOH slightly modifies the affinity of radioligands while a significant diminution of binding sites has been observed. On the contrary a parallel diminution of binding capacity and affinity has been observed when binding is performed in Tes-KOH buffer. This latter result

contrasts with the findings of Barnard and Demoliou-Masson (13). In order to investigate whether tissue differences might explain this discrepancy, we have performed the same experiments on rat brain membranes. Hepes buffer showed similar modifications of binding in the two tissues. On the contrary no significant modification of equilibrium binding parameters was found when Tes-KOH substituted for Tris-HCl buffer. Nevertheless, in the case of ^{3}H EKC binding in which case an heterogeneity of binding sites could be found by mathematical analysis of our data (10), a high affinity component was found in Tris-HCl. This component was not identified in the two other buffer systems.

In both Hepes and Tes buffers, Na^+ and K^+ have been used as counterions. We have therefore further investigated whether the observed variations of binding parameters were due to the effect of these monovalent cations with binding sites (17,18). The results presented in table 1 and 2 do not account for such an explanation. Substitution of K^+ by Na^+ in Tes buffer produced an abolition of binding in the bovine adrenal medulla while more discrete changes were found in the rat brain. This effect cannot be attributed to Na^+ since replacement of this cation with H^+ in the case of Hepes buffer produced a similar effect. We think therefore that the modification of binding parameters can be attributed to a physicochemical interaction of buffer molecules with membranes.

In conclusion, we think that Hepes-NaOH could be substituted for Tris-HCl buffer during opioid binding in the bovine adrenal medulla in thermodynamic or kinetic temperature-dependent studies. Nevertheless the higher number of opioid sites identified in Tris-HCl still makes this latter the buffer of choice in opioid binding studies in the central or the peripheral nervous system.

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