Biochemical Pharmacology, Vol. 36, No. 24, pp. 4257–4262, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

CHARACTERIZATION OF [^{3}H]Ro 5-4864 BINDING TO CALMODULIN USING A RAPID FILTRATION TECHNIQUE

PHILIP F. MORGAN, JITENDRA PATEL and PAUL J. MARANGOS*
Unit on Neurochemistry, Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, MD 20892, U.S.A.

(*Received* 17 *January* 1987; *accepted* 14 *May* 1987)

Abstract—The benzodiazepine [^{3}H]Ro 5-4864 bound specifically and saturably to an apparently homogenous, univalent species of binding site on the calmodulin molecule with an associated equilibrium dissociation rate constant (K_d) of 644 ± 121 nM. The rates of association (K_1) and dissociation (K_{-1}) governing this interaction were estimated to be 7.66×10^3 M^{-1} sec^{-1} and 2.9×10^{-3} sec^{-1}, respectively, yielding a non-equilibrium determination of the K_d to be 379 nM. Such binding of [^{3}H]Ro 5-4864 was protein-, pH-, and temperature-dependent and demonstrated pharmacological selectivity. Only benzodiazepine compounds (chlordiazepoxide, diazepam and Ro 5-4864) inhibited [^{3}H]Ro 5-4864 binding to calmodulin with inhibitory equilibrium dissociation constants (K_i) < 10 μM. The benzodiazepine compounds Ro 15-1788 and flunitrazepam did not displace [^{3}H]Ro 5-4864 binding to calmodulin nor did a number of pharmacologically active non-benzodiazepine compounds (K_i values >10 μM). Consideration of the stoichiometry yielded an approximte mole ratio of 0.90:1.0 (Ro 5-4864:calmodulin), suggesting that there is one binding site for Ro 5-4864 per molecule of calmodulin. The data reveal that the binding of [^{3}H]Ro 5-4864 to calmodulin fulfills the major criteria of a ligand binding to a receptor. Such an interaction may underlie some of the pharmacological actions of Ro 5-4864-like compounds.

The role of calcium as an intracellular messenger of paramount importance is now widely recognized [1]. The ubiquitous Ca^{2+}-binding protein, calmodulin, is thought to play a major role in several of the Ca^{2+}-dependent biological phenomena thus far delineated. These include such diverse and important functions as phosphodiesterase activity [2], activity of adenyl cyclase [3, 4], transmembrane ion transport [5, 6], activity of myosin light chain kinase [7] and the activation of a number of other protein kinases and phosphorylases [8–12]. Calmodulin is also thought to play both a modulatory [13–15] and a direct [14, 16] role in synaptic neurotransmission. Calmodulin thus represents a potentially key target for pharmacological intervention.

Studies of the calmodulin-induced activation of calcium-sensitive phosphodiesterase and other enzymes have revealed that several classes of pharmacological compounds can inhibit calmodulin activity including phenothiazine major tranquilizers [17–22] and benzodiazepine minor tranquilizers [20, 23, 24].

In accord with studies concerning indirect assessment of drug reaction with calmodulin via kinetic studies of enzyme activity, direct dialysis ligand binding studies of tritiated phenothiazine binding to calmodulin have been reported [25–28]. Such binding studies reveal that the phenothiazine major tranquilizer, [^{3}H]trifluoperazine, specifically binds to calmodulin with an affinity constant (K_d) of around 1–2 μM [25–27]. A large number of clinically effective major tranquilizers, including phenothiazine (e.g. chlorpromazine), butyrophenone (e.g. haloperidol), and diphenylbutylpiperidine (e.g. pimozide) compounds, compete with [^{3}H]trifluoroperazine binding to calmodulin with inhibitory dissociation constants (K_i) in the range 1–100 μM [25, 26]. Indeed, on the basis of the rank order of potency of a number of compounds in inhibiting specific [^{3}H]trifluoperazine binding to calmodulin, it has been postulated that interactions with calmodulin may underlie much of the pharmacological profile of action of major tranquilizers [25, 26]. Several other classes of pharmacologically active compounds, including antidepressants (e.g. imipramine), opioid peptides (e.g. β-endorphin) and minor tranquilizers (e.g. diazepam), have also been reported to displace specific [^{3}H]trifluoperazine binding to calmodulin [23, 26, 29]. However, a key problem associated with the assessment of inhibitory dissociation constants as described above using [^{3}H]trifluoperazine is that accurate estimations of these constants are difficult to obtain as a result of the very low signal/noise ratio inherent in the dialysis method employed.

Several lines of evidence suggest that benzodiazepines such as diazepam and particularly the peripheral-type benzodiazepine binding site ligand, Ro 5-4864 [30], may pharmacologically modulate some Ca^{2+}-mediated cellular events such as transmembrane calcium transport [31] and hormone release [32]. In the light of the above lines of evidence suggesting tentative links between benzodiazepines and calmodulin and in view of the key physiological role of calmodulin, we examined the binding of [^{3}H]Ro 5-4864 to calmodulin, using a rapid filtration method.

* Address all correspondence and reprint requests to: Paul J. Marangos, Ph.D., Unit on Neurochemistry, Biological Psychiatry Branch, NIMH, Building 10, Room 3D-48, Bethesda, MD 20892.

MATERIALS AND METHODS

[^{3}H]*Ro* 5-4864 *binding assay.* Calmodulin was incubated (1 hr at 2°) *in vitro* with increasing concentrations of [^{3}H]Ro 5-4864 diluted 1:10 with cold Ro 5-4864 to a specific activity of 8.64 Ci/mmol, and unlabeled ligands in a 50 mM Tris–HCl buffer medium containing 1 mM $CaCl_2$. Non-specific binding was assessed using 10^{-4} M unlabeled Ro 5-4864. At equilibrium, bound ligand was separated from unbound ligand by rapid vacuum filtration of the incubation mixture through Whatman GF/B glass fiber filters pre-soaked in 10% (v/v) polyethylenimine in 50 mM Tris–HCl. Efficiency of the filtration system with respect to calmodulin entrapment was assessed using ^{125}I-calmodulin diluted with calmodulin to the final calmodulin concentration employed in the assays. Radioactivity of tritium samples was counted in 10 ml of scintillation fluid (Beckman Redi-Solv) by scintillation spectroscopy (Beckman LS-2800). ^{125}I-Labeled samples were counted directly by γ-detection (Beckman gamma 4000).

Dialysis of calmodulin. Calmodulin was dialyzed using seamless cellulose dialysis tubing (Fisher, MD) having a molecular weight cut-off of 12,000 daltons as previously described [25]. Calmodulin preparation (4 ml) suspended in 10 mM Tris–HCl, pH 7.0, buffer was dialyzed three times against 4 liters of 10 mM Tris–HCl, pH 7.0, buffer for 8 hr, once in the presence of 1 mM ethyleneglycolbis(amino-ethylether) tetra-acetate (EGTA) and then twice in the absence of EGTA.

Drugs and reagents. [^{3}H]Ro 5-4864 (sp. act. = 86.4 Ci/mmol, 1-*methyl*-^{3}H) and ^{125}I-calmodulin (sp. act. = 50–150 μCi/μg) were purchased from New England Nuclear, Boston, MA. Calmodulin (bovine derived) was purchased from Calbiochem, LaJolla, CA. Homogeneity of the calmodulin preparation was confirmed by analytical gel electrophoresis. All benzodiazepines including Ro 5-4864 (7-chloro-1,3-dihydro-1-methyl-5-[*p*-chlorophenyl]-2H-1,4-benzodiazepine-2 one) and Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4 *H*-imidazo-1,5a-1,4-benzodiazepine-3-carboxylate) were donated by Hoffmann-La Roche, Nutley, NJ. Verapamil and PK11195 (1,2-chlorophenyl)-*N*-methyl-*N*(1-methyl-propyl)-3-isoquinoline carboxamide) were obtained from Knoll Pharmaceuticals, Ludwigshafen, F.R.G., and Pharmuka Laboratories, Gennevilleirs, France, respectively. All other drugs were obtained from Sigma, St. Louis, MO, and reagents were purchased from standard commercial sources.

RESULTS

Employing ^{125}I-calmodulin to monitor efficiency, the total loss of calmodulin throughout the assay (principally by filtration but also by tube adsorption, etc.) was 16.2 ± 0.2% (mean ± SEM). Estimates of efficiency always varied in the range 81–86%, and such estimates were used to correct for system efficiency.

Binding of [^{3}H]Ro 5-4864 to calmodulin exhibited a saturable specific component and an approximately linear non-specific component (Fig. 1A). Plotting saturation isotherms according to the method of Scatchard [33] yielded a linear plot consistent with the binding of a univalent species of ligand to a univalent species of binding site (Fig. 1B). Binding parameters derived from the Scatchard plot were K_d = 442 nM and B_{max} = 28.3 pmol/μg calmodulin. Analysis of a total of six Scatchard plots yielded estimates of (1) 644 ± 121 nM for the equilibrium dissociation rate constant (K_d) describing the affinity of calmodulin for [^{3}H]Ro 5-4864, and (2) 28.0 ± 3.5 pmol/μg calmodulin for the maximum number of

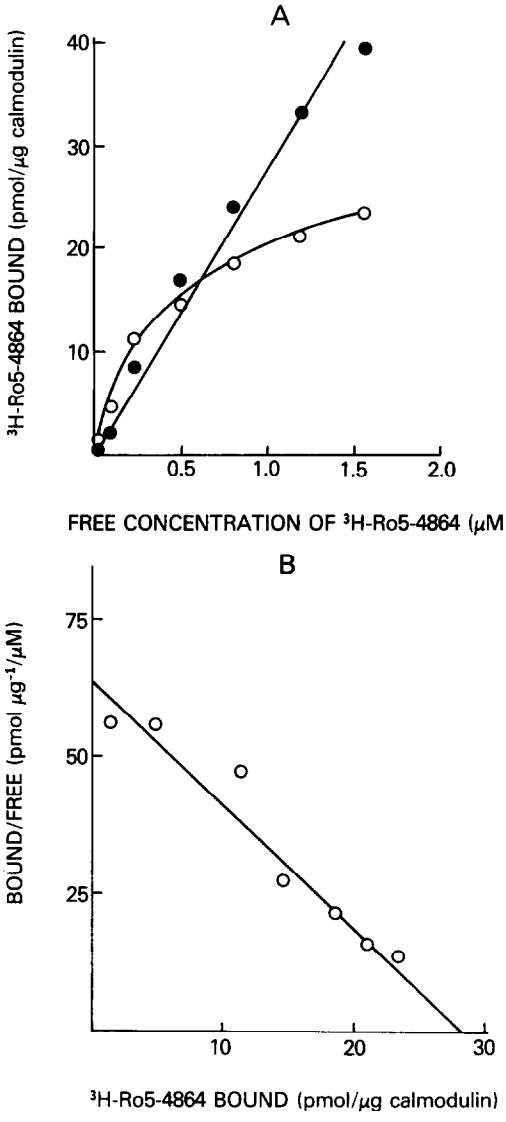


Fig. 1. Saturation of [^{3}H]Ro 5-4864 binding to calmodulin. (A) Calmodulin (80 μg/ml) was incubated with increasing concentrations of [^{3}H]Ro 5-4864 as described in the text. [^{3}H]Ro 5-4864, bound in the presence of 10^{-4} M Ro 5-4864 (non-specific binding, ●), was subtracted from total binding (in the absence of 10^{-4} M Ro 5-4864) to determine specific binding (○). (B) Specific binding replotted according to the method of Scatchard [33]. Data points represent the mean of triplicate determinations from a single experiment.

binding sites for [^{3}H]Ro 5-4864 (Fig. 1B). Since the study employed a purified calmodulin lyophilisate, the value of 28 pmol [^{3}H]Ro 5-4864/μg calmodulin can be employed to estimate approximately the number of drug binding sites per molecule of calmodulin. The extent of impurities (principally salts and buffer) in the purified calmodulin lyphilisate is reported to be 52% by weight (Calbiochem. Co.). Taking this estimate, and using an M_r for calmodulin of 16,700, then 1 μg lyophilized calmodulin extract = 3.1×10^{-11} mol calmodulin. Therefore, there are 28 pmol [^{3}H]Ro 5-4864/3.1×10^{-11} mol calmodulin, which yields an approximate mole ratio of 0.90:1.0 (Ro 5-4864:calmodulin).

Time-course studies confirmed reversibility of binding as well as the appropriateness of employing a 1-hr incubation time, since equilibrium was achieved well prior to this time period (Fig. 2). Analysis of the data on the assumption that association can be described by a pseudo-first-order reaction and dissociation by a first-order reaction yielded an estimate of the association rate constant (K_1) of 7.66×10^3 M^{-1} sec^{-1} and of the dissociation rate constant (K_{-1}) of 2.9×10^{-3} sec^{-1}. Thus, the equilibrium dissociation rate constant (K_d) can be indirectly estimated under non-equilibrium conditions from the ratio K_{-1}/K_1 to be 379 nM for Ro 5-4864 binding to calmodulin. From the dissociation rate constant estimated above, a $T_{1/2}$ of 239 sec can be computed. Since the time period required to filter and wash each sample was in the order of 5 sec, or in terms of $T_{1/2}$ units, 0.02 $T_{1/2}$, it can be estimated that the binding loss due to filtration (i.e. percentage of initial steady-state binding) is less than 2% (see Ref. 34).

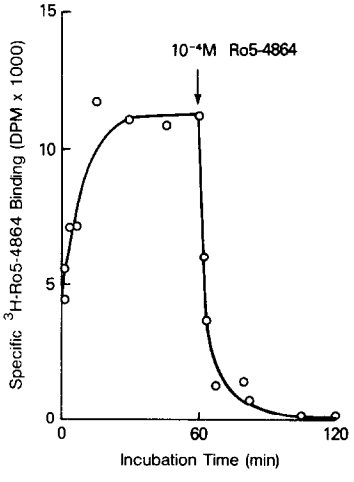


Fig. 2. Association and dissociation of [^{3}H]Ro 5-4864 binding to calmodulin. Calmodulin (80 μg/ml) was incubated with 100 nM [^{3}H]Ro 5-4864, and the amount of total [^{3}H]Ro 5-4864 binding was determined as a function of incubation time by sampling aliquots of a pooled sample after different periods of incubation. At 60-min incubation time, 10^{-4} M Ro 5-4864 was added to the incubation mixture, and the decline in total binding with time was determined as above. Non-specific binding was estimated by processing identical incubates in the presence of 10^{-4} M Ro 5-4864. Specific binding was estimated from the difference (total minus non-specific binding). Data points represent the mean of triplicate determinations from a single experiment. Two further experiments yielded similar results.

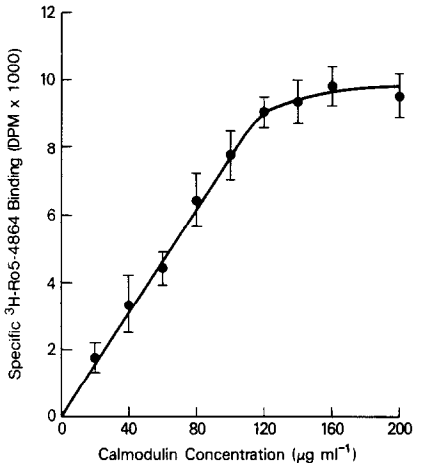


Fig. 3. Specific [^{3}H]Ro 5-4864 binding to calmodulin as a function of protein concentration. [^{3}H]Ro 5-4864 was incubated with calmodulin at different protein concentrations as described in the text. Non-specific binding was assessed using 10^{-4} M Ro 5-4864. Data points represent the mean ± SEM of three experiments performed in duplicate.

The binding of [^{3}H]Ro 5-4864 to calmodulin was found to be both temperature and pH dependent with optimal binding observed at 2° and pH 7 (data not shown). The binding was also dependent upon protein concentration with the amount of specific binding varying linearly as a function of calmodulin concentration up to a calmodulin concentration of approximately 120 μg/ml (Fig. 3).

Commercial calmodulin is supplied in a medium containing calcium ions. The addition of further calcium ions (up to a concentration of 100 mM in the form of $CaCl_2$) caused only small (<10%) increases in specific [^{3}H]Ro 5-4864 binding (data not shown). However, after dialyzing calmodulin against an EGTA buffer to obtain Ca^{2+}-free calmodulin, poten-

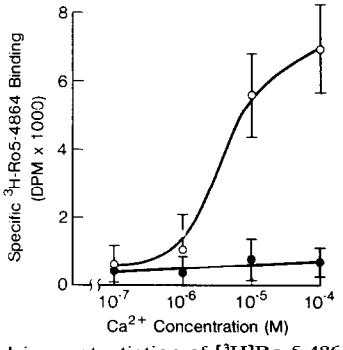
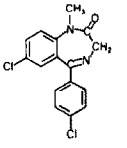
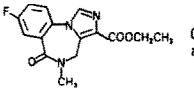
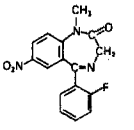
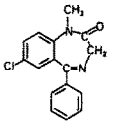
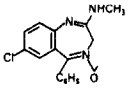
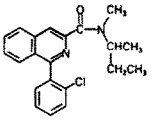
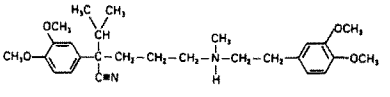
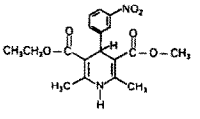
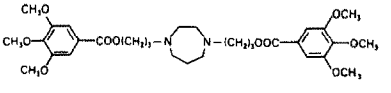
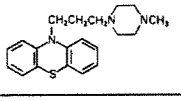


Fig. 4. Calcium potentiation of [^{3}H]Ro 5-4864 binding to calmodulin. Dialyzed calmodulin (80 μg/ml) was incubated with [^{3}H]Ro 5-4864 as described in the text with increasing concentrations of $CaCl_2$ in the absence (○) and presence (●) of 10 mM EDTA. Non-specific binding was assessed using 10^{-4} Ro 5-4864. Data points represent the mean ± SEM of four experiments performed in triplicate.

tiation of specific [^{3}H]Ro 5-4864 binding to calmodulin was demonstrated; the potentiation was prevented by EGTA (Fig. 4). Non-specific binding of [^{3}H]Ro 5-4864 was not altered by either Ca^{2+} or EGTA (data not shown).

Displacement studies revealed that the binding of [^{3}H]Ro 5-4864 to calmodulin showed ligand selectivity (Table 1). Of the compounds studied, only benzodiazepine compounds inhibited [^{3}H]Ro 5-4864 binding with K_i values $< 10\ \mu M$. These were chlor-

Table 1. Inhibition of [^{3}H]Ro 5-4864 binding to calmodulin

COMPOUND	STRUCTURE	PHARMACOLOGICAL DESCRIPTION	INHIBITORY EQUILIBRIUM DISSOCIATION CONSTANT, K_i (nM)
RO 5-4864		Peripheral-type benzodiazepine	681 ± 158
RO 15-1788		Central-type benzodiazepine antagonist	>10,000
Flunitrazepam		Central-type benzodiazepine	>10,000
Diazepam		Mixed central/peripheral type benzodiazepine	1600 ± 100
Chlordiazepoxide		Mixed central/peripheral type benzodiazepine	3200 ± 190
PK11195		Triazolopyridazine peripheral-type benzodiazepine binding site ligand	>10,000
Verapamil		Calcium channel blocking agent (non-dihydropyridine)	>10,000
Nitrendipine		Calcium channel blocking agent (dihydropyridine)	>10,000
Dilazep		Nucleoside uptake inhibitor	>10,000
Trifluoperazine		Phenothiazine major tranquilizer	>10,000

Test compound and [^{3}H]Ro 5-4864 were incubated with calmodulin (80 μg/ml) as described in the text. K_i values were determined from the concentration of inhibitor which caused 50% inhibition of specific binding (IC_{50}) (obtained by linear regression analysis of log probit plots of at least six concentrations) using the equation of Cheng and Prusoff [35], employing the previously determined K_d of 644 nM obtained by Scatchard analysis (Fig. 1). Data represent the mean ± SEM of at least three experiments.

diazepoxide, diazepam and Ro 5-4864. However, the benzodiazepine compounds Ro 15-1788 and flunitrazepam did not displace [^{3}H]Ro 5-4864 binding, nor did the peripheral-type benzodiazepine binding site ligand, PK11195 (K_i values $> 10\ \mu M$). Several other classes of compounds including calcium channel blocking agents (verapamil and nitrendipine), a nucleoside uptake inhibitor (dilazep), and a major tranquilizer (trifluoperazine) were also found not to displace [^{3}H]Ro 5-4864 binding to calmodulin (K_i values $> 10\ \mu M$) (Table 1).

DISCUSSION

The main conclusion of the present study is that the binding of [^{3}H]Ro 5-4864 to calmodulin fulfilled the major criteria of ligand binding to a receptor. That is to say, it was saturable, reversible, pH-, protein- and temperature-dependent, and exhibited ligand selectivity. The data, therefore, demonstrate direct binding of a benzodiazepine ligand to calmodulin. Since stoichiometric consideration revealed an approximate mole ratio of 0.90:1.0, this suggests that there is one binding site for Ro 5-4864 per molecule of calmodulin.

Despite the relatively low affinity of this interaction, separation by rapid filtration was technically very feasible in view of the relatively slow dissociation rate. The maximum allowable separation time for a ligand binding assay is widely accepted to be around 0.15 $T_{1/2}$ [34] in order to allow a loss of not more than 10% bound ligand during separation. In the present study, this corresponds to an upper limit for separation of approximately 36 sec—a considerably longer time period than was actually required (viz. approximately 5 sec). In fact, taking the separation time to be 5 sec, the binding loss due to filtration was <2%. The presently described method, therefore, may be viewed as an alternative to the widely used trifluoperazine/dialysis method in analytical protocols. Furthermore, in view of the inherent disadvantages of a very low signal/noise ratio and extremely lengthy procedures of protocols involving ligand (phenothiazine) binding to calmodulin by dialysis, the present method involving a 60-min incubation/rapid filtration technique of studying ligand binding to calmodulin can be viewed as a considerable advance over previous methods of studying ligand binding to calmodulin.

One of the salient features of the pharmacological specificity of the binding site presently studied is that the central-type benzodiazepine receptor ligands, flunitrazepam and Ro 15-1788, are not active in displacing [^{3}H]Ro 5-4864 binding to calmodulin. This would suggest that inhibition of calmodulin activity does not play a major role in underlying the pharmacological action of the minor tranquilizer, flunitrazepam, or the central-type benzodiazepine antagonist, Ro 15-1788. Despite the potency of the peripheral-type ligand, Ro 5-4864, and the mixed (peripheral/central)-type benzodiazepine binding site ligands (diazepam, chlordiazepoxide) in inhibiting [^{3}H]Ro 5-4864 binding to calmodulin, the pharmacological specificity of the calmodulin binding site for Ro 5-4864 is not similar to the pharmacological specificity of the peripheral-type benzodiazepine binding site since the peripheral-type benzodiazepine binding site antagonist ligand, PK11195, was not active in displacing specific [^{3}H]Ro 5-4864 binding to calmodulin. Such an observation would tend to preclude a common identity between the peripheral-type benzodiazepine binding site and the calmodulin binding site for Ro 5-4864. The lack of potency of PK11195 also suggests that modulation of calmodulin activity is only a tenable hypothesis to explain the pharmacological effects of Ro 5-4864 that are insensitive to PK11195.

It is widely considered that pharmacological modulation of calmodulin function is achieved by inducing changes in the tertiary structure of calmodulin. Since Ro 5-4864 is a detectable displacer of [^{3}H]trifluoperazine binding to calmodulin and yet trifluoperazine did not detectably displace [^{3}H]Ro 5-4864 binding to calmodulin at a concentration considerably above the reported K_d for [^{3}H]trifluoperazine binding to calmodulin [25–27], Ro 5-4864 may well bind to a different loci on the calmodulin molecule to trifluoperazine and displace [^{3}H]trifluoperazine binding to calmodulin by (non-reciprocal) allosteric hindrance. The possibility of two or more modulatory sites on the calmodulin molecule is of considerable pharmacological interest because it is already known that there are two regions on calmodulin which are capable of interacting with target proteins (kinases and phosphorylases), one on the N-terminal moiety and another on the C-terminal moiety [36]. Pharmacological intervention at disparate modulatory sites, therefore, may very well elicit different biochemical profiles of action of calmodulin.

REFERENCES

1. F. C. Stevens, *Can. J. Biochem. Cell. Biol.* **61**, 906 (1983).
2. P. Uzonov and B. Weiss, *Biochim. biophys. Acta* **284**, 220 (1972).
3. K. B. Seamon and J. W. Daly, *Life Sci.* **30**, 1457 (1982).
4. G. T. Peake and J. A. Smoake, *Endocrinology* **116**, 2098 (1985).
5. R. M. Levin and B. Weiss, *Neuropharmacology* **19**, 169 (1980).
6. F. F. Vincenzi, T. J. Andreasen and T. R. Hinds, in *Calcium and Phosphate Across Biomembranes* (Eds. F. Bronner and M. Peterlik), pp. 45–50. Academic Press, New York (1981).
7. K. Yagi, M. Yazawa, S. Kakiuchi, M. Ohshima and K. Uenishi, *J. biol. Chem.* **253**, 1338 (1978).
8. P. Nose and H. Schulman, *Biochem. biophys. Res. Commun.* **107**, 1082 (1982).
9. S. Kakiuchi, *Neurochem. Int.* **3**, 159 (1983).
10. T. L. McGuiness, Y. Lai, P. Greengard, J. R. Woodgett and P. Cohen, *Fedn Eur. Biochem. Soc. Lett.* **163**, 329 (1983).
11. D. M. Watterson, W. H. Burgess, T. J. Lukas, D. Iveron, D. R. Marshak, M. Schleicher, B. W. Erickson, K. F. Fok and L. J. Eldik, in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Eds. S. J. Strada and W. J. Thompson), Vol. 16, pp. 116–21. Raven Press, New York (1984).
12. M. L. Billingsley, P. A. Velletri, W. Lovenberg, D. Kuhn, J. R. Goldenring and R. J. DeLorenzo, *J. Neurochem.* **44**, 1442 (1985).
13. R. J. DeLorenzo, *Ann. N.Y. Acad. Sci.* **356**, 92 (1980).

14. R. J. DeLorenzo, *Fedn Proc.* **41**, 2265 (1982).
15. S. Richardson and S. Twenke, *Endocrinology* **117**, 369 (1985).
16. R. J. DeLorenzo, *Cell Calcium* **2**, 365 (1981).
17. B. Weiss, R. Fertel, R. Figlin and P. Uzunov, *Molec. Pharmac.* **10**, 615 (1974).
18. R. M. Levin and B. Weiss, *Molec. Pharmac.* **12**, 581 (1976).
19. M. Volpi, R. I. Sha'afi, P. M. Epstein, D. M. Andrenyak and M. B. Feinstein, *Proc. natn. Acad. Sci. U.S.A.* **78**, 795 (1981).
20. W. C. Prozialeck and B. Weiss, *J. Pharmac. exp. Ther.* **222**, 509 (1982).
21. R. W. Wrenn, N. Katol, R. C. Schatzman and J. F. Kuo, *Life Sci.* **29**, 725 (1981).
22. R. J. DeLorenzo and J. R. Goldenring, in *Brain Receptor Methodologies* (Eds. P. J. Marangos, I. C. Campbell and R. M. Cohen), pp. 191–207. Academic Press, New York (1984).
23. B. Weiss, W. C. Prozialeck, M. S. Barnette and T. L. Wallace, *Ann. N.Y. Acad. Sci.* **356**, 319 (1980).
24. R. J. DeLorenzo, S. Burdette and J. Holderness, *Science* **213**, 546 (1981).
25. R. M. Levin and B. Weiss, *Molec. Pharmac.* **13**, 690 (1977).
26. R. M. Levin and B. Weiss, *Biochim. biophys. Acta* **540**, 197 (1978).
27. R. M. Levin and B. Weiss, *J. Pharmac. exp. Ther.* **208**, 454 (1979).
28. B. Weiss, *Meth. Enzym.* **102**, 171 (1983).
29. M. S. Barnette and B. Weiss, *Molec. Pharmac.* **21**, 86 (1982).
30. C. Braestrup and M. Nielsen, *Trends Neurosci.* **3**, 301 (1980).
31. S. M. Paul, M. D. Luu and P. Skolnick, in *Pharmacology of the Benzodiazepines* (Eds. E. Usdin, P. Skolnick, J. F. Tallman, D. Greenblatt and S. M. Paul), pp. 87–92. Macmillan Press, New York (1983).
32. J. C. Bisserbe, J. Patel and R. L. Eskay, *J. Neurochem.* **47**, 1419 (1986).
33. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
34. J. P. Bennett and H. I. Yamamura, in *Neurotransmitter Receptor Binding* (Ed. H. I. Yamamura), 2nd Edn, pp. 61–89. Raven Press, New York (1985).
35. Y. C. Cheng and W. H. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
36. D. Newton, C. B. Klee, J. R. Woodgett and P. Cohen, *Biochim. biophys. Acta* **845**, 533 (1985).