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## Accelerated Publications

# Identification of an Opioid Receptor Subunit Carrying the $\mu$ Binding Site†

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ABSTRACT: The enkephalin affinity reagent [$^3$H]Tyr-D-Ala-Gly-Phe-Leu-$CH_2Cl$ ([$^3$H]DALECK) was synthesized. It exhibited high-affinity reversible binding, at pH 7.4, to both $\mu$ and $\delta$ opioid receptor sites in rat brain membranes. At pH 8.1, nanomolar levels of [$^3$H]DALECK produced an irreversible labeling in synaptic membranes, essentially only in one subunit of 58 000 daltons. The irreversible phase of the reaction reduced the subsequent binding of a $\mu$-selective enkephalin derivative but not that of a $\delta$-selective one. It is concluded that a $\mu$ subunit of the opioid receptor exists, can be alkylated specifically, and is of $M_r$ 58 000.

It has become well established, both from pharmacological and from membrane ligand-binding approaches, that the opioid receptor system encompasses multiple subtypes, i.e., $\delta$, $\mu$, $\kappa$ (Paterson et al., 1983; Zukin & Zukin, 1984), and perhaps others, but the molecular basis of this receptor heterogeneity has remained obscure. Some progress has been made in the solubilization of active, high-affinity opioid-binding sites from mammalian brain, e.g., for opiate sites in digitonin/1 M NaCl (Howells et al., 1982) or for these and opioid peptide $\delta$, $\mu$, and and $\kappa$ sites in digitonin/10 mM $MgCl_2$ (Demoliou-Mason & Barnard, 1984). However, the relationship between receptor subunit structure and these multiple types of binding sites has not been clarified. The only clear-cut evidence so far in this direction has been for a subunit of the receptor with the $\delta$ type of specificity found in the neuroblastoma x glioma (NG108-15) cell line, which was recognized by affinity labeling with the pyridinium derivative [$^3$H]fentanyl isothiocyanate by Klee et al. (1982). This reaction produced a labeled subunit of $M_r$ 58 000. In another such approach, an opioid pentapeptide carrying an alkylating function at its C-terminus was sought; for such a modification of a peptide the chloromethyl ketone group is particularly suitable (Shaw, 1967), and in the first stage of this work, D-Ala$^2$-Leu-enkephalin chloromethyl ketone (DALECK)[1] was synthesized and evidence found for its production of a specific, irreversible inhibition of opioid receptors, both in organ-bath bioassay and in brain membrane ligand binding (Venn & Barnard, 1981). The synthesis of DALECK and its irreversible blockade of opiate receptor sites have since been confirmed by Szucs et al. (1983), who further showed that about 50% of the [$^3$H]naloxone binding sites in brain membranes can be irreversibly and specifically blocked by this reagent. We report here more evidence on the specificity of DALECK and employ it in tritiated form to label an individual subunit of the brain opioid receptor.

## Materials and Methods

*Materials.* Boc($I_2$)-Tyr-D-Ala-Gly was a gift from Dr. M. J. Rance (then of Reckitt and Colman Ltd., Hull, England). The halogen in this intermediate was exchanged for tritium over a palladium catalyst at the laboratories of Amersham International plc. The product was at least 95% pure (as estimated by thin-layer chromatography on silica in chloroform/ethanol, 9:1 v/v) and had a specific activity of 2.64 Ci mmol$^{-1}$. *N*-Methylmorpholine and isobutyl chloroformate were from Aldrich; trifluoroacetic acid and methanol were analytical-grade reagents from BDH. Other chemicals were from Sigma or were as specified by Venn & Barnard (1981).

*Synthesis of [$^3$H]DALECK.* Boc-[$^3$H]Tyr-D-Ala-Gly (0.55 mmol) was evaporated from anhydrous THF to dryness and dissolved in 17 $\mu$L of THF; a 3-fold molar excess of *N*-methylmorpholine was added in a further 2 $\mu$L of THF. The glass microvessel was chilled to −20 °C, and a 1.4-fold excess

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; DADLE, D-Ala$^2$-D-Leu$^5$-enkephalin; DAGO, Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol; DALECK, D-Ala$^2$-Leu-enkephalin chloromethyl ketone; DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; TPCK, *N*-tosylphenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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of isobutyl chloroformate was added. After 10-min reaction, there was added a 1.3-fold excess (0.77 mmol) of Phe-Leu-$CH_2Cl$, prepared and characterized according to Venn & Barnard (1981). After standing for 90 min at −20 °C and then 1 h at 4 °C, the product was evaporated to a film, dissolved in 40 μL of freshly double-distilled trifluoroacetic acid, left at room temperature for 50 min to remove the Boc group, and lyophilized twice from methanol. More than 95% of the starting radioactivity was recovered in the final involatile product.

This crude product was stored in dry methanol at −20 °C and purified in batches by preparative HPLC immediately before use. A sample (50 μL) was applied to an Altex Ultrasphere ODS 250 × 10 mm reverse-phase HPLC column and eluted (2.5 mL $min^{-1}$) under isocratic conditions in methanol/water/trifluoroacetic acid (630:360:0.4 v/v). Aliquots (5 μL) from each 1.25-mL fraction were counted by liquid scintillation. The product in peak b (Figure 1) comigrated with authentic nontritiated DALECK (Venn & Barnard, 1981) on silica thin-layer chromatograms developed in (i) chloroform/ethanol, 9:1, (ii) chloroform/methanol/acetic acid, 8:3:1, and (iii) ethyl acetate/pyridine/acetic acid/water, 50:20:6:11 ($R_f$ values of 0.19, 0.85, and 0.93, respectively).

*Assays of Ligand Binding.* A crude synaptic/mitochondrial/microsomal membrane fraction from rat forebrain, prepared in the presence of a set of protease inhibitors as described elsewhere (Lai et al., 1984), was used, except where gradient-purified synaptic plasma membranes are specified. The latter were prepared (but with the addition of the same protease inhibitors) and characterized according to Jones & Matus (1974). Membranes were incubated at 25 °C in a quasi-physiological buffer [buffer T: 100 mM NaCl, 15 mM $Na_2HPO_4$, 3 mM KCl, 2 mM $MgCl_2$, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, containing the protease inhibitors but omitting phenylmethanesulfonyl fluoride]. Also present at this stage were 3 μM TPCK (shown in tests to give no change in the binding of [$^3$H]DAGO or [$^3$H]DADLE) and any nonradioactive ligands used (e.g., 2 μM etorphine to measure nonspecific binding or various concentrations of other opiates to examine competition with the radioligand). After 30 min, the tritiated ligand was added to give the stated final concentration in a final volume of 1 mL. The membrane protein concentration (Markwell et al., 1978) in these reactions was 1 mg $mL^{-1}$. After an incubation with [$^3$H]DALECK for 10 min, or [$^3$H]DAGO or [$^3$H]DADLE for 30 min at 25 °C, the membranes were rapidly filtered on Whatman GF/C glass fiber disks (2.4-cm diameter) and the disks washed with 2 × 4 mL of ice-cold 50 mM Tris-HCl (pH 7.4), dried, and counted.

*Affinity Labeling of Brain Membranes.* The membranes were suspended in buffer T at a protein concentration of 1.0–1.5 mg $mL^{-1}$, with TPCK also present at 3 μM. After 30 min at 25 °C, [$^3$H]DALECK was added to 30 nM concentration and after 4 min aliquots (1 mL) were filtered through GF/C disks and the disks washed with 2 × 4 mL of buffer T and then with 2 × 4 mL of 50 mM disodium tetraborate hydrochloride (pH 8.1). The damp filters were further incubated at 25 °C for 1 h and then washed with 5 × 4 mL of 20 mM sodium acetate/120 mM NaCl/1 mM EDTA (pH 4.5), 2 × 4 mL of water, and 2 × 4 mL of methanol. The filters were dried under an infrared lamp. Membrane proteins were completely extracted from the filters with 1 mL of 2% SDS/10 mM Tris-HCl (pH 8.8)/1 mM EDTA/0.3% DTT solution (preheated to 80 °C). A sample was removed for determination of protein (Markwell et al., 1978) and radioactivity, and the extract was lyophilized and used for gel electrophoresis. Nonspecific labeling was determined by carrying out a duplicate experiment, but including 2 μM etorphine in the initial incubation of the membranes with TPCK.

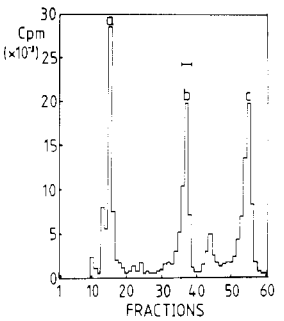


FIGURE 1: Purification of [$^3$H]DALECK by reverse-phase HPLC. The solvent front is at fraction 10. The bar above peak b indicates the elution zone of pure nonradioactive DALECK.

## Results and Discussion

*Characterization of [$^3$H]DALECK.* The method of Venn & Barnard (1981) was modified and scaled down about 300-fold in order that excessive amounts of radioactivity were not required. It proved convenient to iodinate the phenol group in Boc-Tyr-D-Ala-Gly, exchange the halogen for $^3$H, and couple the tritiated product to preformed Phe-Leu-$CH_2Cl$. Fractionation by reverse-phase HPLC of the crude [$^3$H]DALECK obtained thus revealed about 20% of the starting radioactivity eluting at the position of authentic DALECK (peak b, Figure 1). Another 30% was identified as the unconjugated [$^3$H]Tyr-D-Ala-Gly (peak a). At least two other radioactive species, more strongly retarded on the column than DALECK, were also formed. Peak c (Figure 1) may represent radioactive Tyr-D-Ala-Gly bound to more than one Phe-Leu chloromethyl ketone moiety; an increase in the ratio of the latter to the former only decreased the yield. Reaction on this small scale is extremely sensitive to moisture, and despite the drying of all the materials used, this would account for the 30% failure of the coupling reaction at the optimal ratio.

[$^3$H]DALECK, as isolated by this HPLC method, appeared to be homogeneous. It comigrated with the nonradioactive DALECK (itself 99% pure by fast-atom bombardment mass spectrometry and with the predicted fragmentation pattern) not only in the HPLC system of Figure 1 but also in three thin-layer chromatographic systems (see Materials and Methods), without any trace of other spots. (The HPLC system was capable of resolving very similar enkephalin derivatives; e.g., DALECK could be clearly distinguished from its bromomethyl ketone analogue, synthesized by equivalent procedures, the chloro and bromo forms having retention times of 10.0 and 12.6 min, respectively, when run on an analytical ODS column at 0.6 mL $min^{-1}$, all other conditions being as described.) It also cochromatographed with the authentic DALECK on a Sephadex LH-20 column equilibrated and eluted in 1 M aqueous acetic acid, as the sole radioactive product.

*Binding to Brain Membranes at pH 7.4.* From initial experiments it was clear that DALECK is an extremely hydrophobic peptide, as expected from the loss of the carboxyl and the addition of the $-CH_2Cl$ group. Consequently, it produced a high level of binding to, and possibly alkylation of, nonspecific sites on the brain membranes used. The conditions described under Materials and Methods represent the optimum found in extensive trials, with exposure to the solution

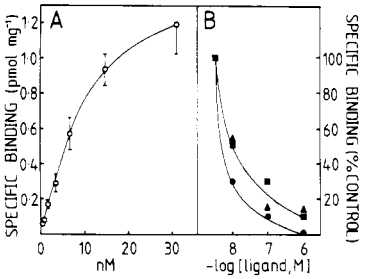


FIGURE 2: Membrane-binding properties of [$^3$H]DALECK. In all cases nonspecific binding was measured by including 2 $\mu$M etorphine in the preincubation and assay stages and was subtracted. (A) Specific binding as a function of [$^3$H]DALECK concentration (nanomolar). Each point is the mean of a triplicate assay; bars indicate standard deviations. (B) Competition of [$^3$H]DALECK (2 nM) with nonradioactive opiate ligands: ●, etorphine; ■, DADLE, ▲, DAGO. The specific binding of 2 nM [$^3$H]DALECK alone was taken as 100%.

of the peptide being kept to a minimum length of time (shown to be sufficient to allow almost maximal specific binding to develop) and with pretreatment of the membranes with the reagent TPCK (Shaw, 1967) so as to block at least some of the nonspecific sites reactive to chloromethyl ketones. Under these conditions, the nonspecific component (measured in the presence of 2 $\mu$M etorphine) of the membrane-bound radioactivity could be restricted to about 50% of the total (at concentrations of radioactive DALECK of around 10 nM).

The specific binding of [$^3$H]DALECK at pH 7.4 to a crude synaptic–microsomal membrane preparation from rat brain increased with the reagent concentration and showed an apparent approach to saturation at around 30 nM (Figure 2A). A complete saturation curve could not be generated, because of the extremely high (~80%) nonspecific binding observed above 30 nM reagent. In the conditions used here (pH 7.4, 10-min reaction) the *reversible* phase of DALECK binding is being monitored; when tested after 2 or 30 nM [$^3$H]DALECK reaction, but with a subsequent repeat of the washes on the filter using instead sodium acetate buffer at pH 4.5, 25 °C (conditions that would remove all binding of [$^3$H]DADLE etc.), this latter wash removed 100% (at 2 nM) or 95% (at 30 nM) of the specific binding of [$^3$H]DALECK. The specific binding was reduced with high potency by both $\delta$- and $\mu$-directed opioid ligands (Figure 2B). The general opioid agonist etorphine gave greater protection than did the $\mu$-preferring peptide DAGO or the $\delta$-preferring peptide DADLE, in line with the known affinities of these ligands in rat brain (Gillan & Kosterlitz, 1982). The residual 10% binding seen at 1 $\mu$M DADLE or DAGO, though statistically not significantly different from the nonspecific binding, may be attributed to a small, irreversible component of the DALECK reaction in the conditions used. The results shown in Figure 2 are consistent with the previous finding (Venn & Barnard, 1981) of an $IC_{50}$ of 5 nM for the displacement of [$^3$H]Leu-enkephalin (when present at 2 nM) by unlabeled DALECK on rat brain membranes in Tris buffer, pH 7.4, at 25 °C. The results overall suggest that both $\mu$ and $\delta$ sites participate in the reversible high-affinity binding of DALECK.

*Membrane Components Alkylated by DALECK.* Purified synaptic plasma membranes were used in these experiments. These membranes were as before, pretreated with the nonopioid chloromethyl ketone, TPCK (at 3 $\mu$M), a treatment that gave no loss of $^3$H-labeled opioid binding [as also noted previously (Venn & Barnard, 1981)] but which considerably reduced the nonspecific reaction of [$^3$H]DALECK. The membranes were then exposed briefly to 30 nM [$^3$H]DALECK at pH 7.4 and the excess reagent was removed by filtration and washing as in the reversible binding assay, but the pH was then raised to 8.1 and the damp membranes were permitted to react on the filters for 1 h. Permitting the reaction to occur only with the bound ligand, in this manner, gave a major reduction in the nonspecific uptake of radioactivity from [$^3$H]DALECK, as compared with the level found if the reaction was conducted for the same total period with the free $^3$H ligand present throughout. In several comparisons made thus, the reduction in nonspecific irreversible binding due to both the measures noted here was of the order of 90%, which permitted specifically alkylated components to be readily detected. Alkylation on the filters was quenched and reversibly bound $^3$H ligand removed (see above) by changing the pH to 4.5 and extensive washing at that pH. This procedure gave a specific uptake of [$^3$H]DALECK into synaptic membrane proteins of 110 fmol/mg (mean of three experiments) after full extraction with 2% SDS. Overnight dialysis of this soluble material against a medium containing 0.2% SDS did not reduce its radioactive content, suggesting a covalent interaction of [$^3$H]DALECK to protein of 110 fmol/mg of protein. The change of the reaction pH from 7.4 to 8.1, with all of the procedure otherwise identical in both cases, was shown to be itself responsible for a 2.8-fold increase in the specific irreversible uptake.

The alkylated membrane components were analyzed by SDS–polyacrylamide gel electrophoresis. A comparison was made by reacting and analyzing similarly the crude membrane fraction used previously. In the latter, the gels showed (Figure 3, lower panel) specific labeling in (i) a strongly labeled band at $M_r$ 58 000, (ii) a diffusely labeled region at $M_r$ 100 000–110 000, perhaps representing an incompletely reduced dimer of the 58 000 species, and (iii) a weakly and variably labeled region at $M_r$ 42 000–48 000. This last may arise from proteolysis of the $M_r$ 58 000 polypeptide or may contain another, much less reactive opiate-binding subunit.

When the purified synaptic plasma membranes were used instead, the $M_r$ 58 000 species was the only prominent product (Figure 3, upper panel); products ii and iii were present but at low levels only. The main difference in opioid receptor sites between the two membrane preparations from mammalian brain is the additional presence of these receptors on microsomal membranes in the crude preparation; these contain in the range of 30–60% of the total high-affinity sites for several types of opioid ligand (Glasel et al., 1980; Roth & Coscia, 1984). This "microsomal" fraction appears to include dendritic and extrasynaptic plasma membranes, as well as Golgi and smooth endoplasmic reticular membranes. The present results show that the synaptic and nonsynaptic opioid receptors have DALECK-reactive components of the same size.

To examine the selectivity of DALECK in its irreversible reaction, crude membranes were incubated with 30 nM nonradioactive DALECK under conditions identical with those just described for the [$^3$H]DALECK reaction at pH 8.1. The reaction was quenched with 5 mM DTT in buffer T at pH 7.4, and the membranes were washed thoroughly, with three centrifugations and resuspensions in that medium, so as to remove all the excess DALECK without inactivating receptors in the process. The remaining opiate-binding sites were then assayed in buffer T, as described under Materials and Methods, with [$^3$H]DAGO for the $\mu$ sites or [$^3$H]DADLE for the $\delta$ sites. Scatchard plots of the data (Figure 4) showed that DALECK caused a permanent reduction in the number of high-affinity DAGO-binding sites, with rather little effect on the affinity of the sites for this ligand. The number of DAGO-binding sites that had become blocked, as shown by

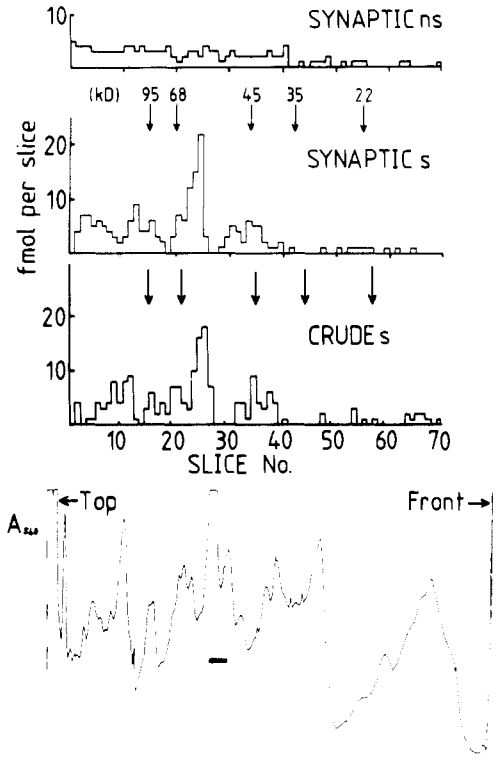


FIGURE 3: SDS–polyacrylamide gel electrophoresis of membranes alkylated with [$^3$H]DALECK. After alkylation at pH 8.1, quenching, and extraction in hot SDS/DTT solution (see Materials and Methods), a sample (0.5 mg of protein) was applied to a stacking gel above the 15 × 0.5 cm 10% polyacrylamide gel; the Laemmli (1970) procedures were used at 3 mA per tube. The positions of markers are shown by the arrows with their $M_r$ values. Radioactive gels were sliced (2 mm) and the $^3$H content was determined. Control samples, alkylated under identical conditions but with 2 $\mu$M etorphine present before and during the [$^3$H]DALECK reaction, were run in parallel rods. The nonspecific level of $^3$H in each slice, obtained thus, was subtracted to plot the specific label per slice. From the top down are displayed the nonspecific (control) labeling of synaptic plasma membranes, the specific labeling of synaptic plasma membranes, and the specific labeling of the crude membranes. Also shown is a scan of a parallel gel, loaded with one-tenth the amount of protein from a crude membrane preparation, stained with Coomassie blue, and scanned at 540 nm. The position to which the major [$^3$H]DALECK-labeled species ran in a parallel gel is denoted by the horizontal bar.

the extrapolated maximal binding values for these high-affinity sites, amounted to about 130 fmol mg$^{-1}$. This is in reasonable agreement with the amount of specific irreversible labeling, noted above, in the [$^3$H]DALECK reaction at the same concentration. The Scatchard plot for the binding of [$^3$H]DADLE was unchanged by the DALECK pretreatment (not shown). For example, at 20 nM [$^3$H]DADLE, specific binding to pretreated membranes was 97 ± 7% (mean ± standard deviation for three determinations) of that to control membranes. The fact that only about 30% of the DAGO-binding sites are occluded (Figure 4) is simply a measure of the partial extent to which the reaction was permitted to proceed (by the withdrawal of the bathing [$^3$H]DALECK solution at the start) so as to minimize nonspecific alkylation. Similarly, the fact that no significant loss of DADLE binding occurs, despite the known cross-reaction of this ligand with $\mu$ sites (Gillan & Kosterlitz, 1982), is accounted for by the combination of this partial reaction of DALECK with $\mu$ sites and the 8-fold weaker binding of DADLE to $\mu$ sites relative to $\delta$ sites (Lutz et al., 1984). At the concentrations of [$^3$H]DADLE used (up to 20 nM), these two factors lead to the prediction that only minimal reduction (less than the degree of experimental variation) in the binding of this ligand would be observed.

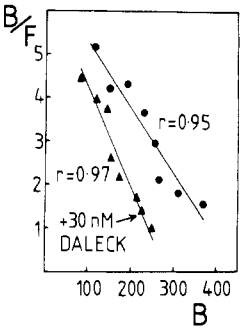


FIGURE 4: Scatchard plots for specific binding of [$^3$H]DAGO. $B$ = bound ligand in femtomoles per milligram of protein; $B/F$ = bound/free ligand in units of $10^5$ liters per milligram. Nonspecific binding was measured in the presence of 2 $\mu$M etorphine. The correlation coefficients for the least-squares lines are marked; only the high-affinity set of sites was used in fitting the data. The crude membranes used had previously been treated as described with (▲) or without (●) 30 nM DALECK and incubated at pH 8.1 and 25 °C for 1 h. The free and reversibly bound ligand was removed, as described in the text, before assaying with [$^3$H]DAGO. In several experiments conducted at 16 nM [$^3$H]DAGO, binding after treatment with DALECK was 77% of the control. Analysis of the data by Student's $t$ test shows this reduction to be highly significant ($p \ll 0.01$, four experiments, each in triplicate).

These results show that an $M_r$ 58 000 species reacts specifically and covalently with DALECK and suggest that this represents a subunit of the $\mu$ subtype of the opiate receptor. We can reconcile this with the evidence for the reversible high-affinity binding of DALECK at both $\mu$ and $\delta$ sites observed in membrane experiments (Figure 2B) and in bioassay of the compound or its metabolic derivatives (Pelton et al., 1980; Venn & Barnard, 1981; Szucs et al., 1983) if a $\delta$-binding subunit exists and binds DALECK, also, but does not have an alkylatable target for this reagent in the same position as in the $\mu$ subunit. The other types of observation previously made on DALECK were with much higher concentrations thereof; its effectiveness at nanomolar concentrations, exemplified in the present work, testifies to a specific reaction with the opiate receptor.

The fact that the subunit of the $\delta$-binding type of receptor, which is present in NG108-15 cells, labeled by fentanyl isothiocyanate (Klee et al., 1982), and the subunit of the $\mu$ subtype in rat brain labeled by DALECK have identical sizes encourages the idea of homology between opiate receptor subtypes. Data from our and another laboratory on the radiation inactivation of the opiate receptor subtypes have, likewise, shown that the $\mu$ and $\delta$ subtypes have equal sizes (Lai et al., 1984; Ott et al., 1983). The target size in those two studies, in both rat brain and NG108-15 cells, corresponds to an $M_r$ of 100 000–110 000, compatible with a dimer of the $M_r$ 58 000 species detected in SDS–polyacrylamide gels (Figure 3).

The preparative method we have used can also yield [$^3$H]DALECK at much higher specific radioactivity, by working initially with a greater amount of tritium. We are currently performing this preparation, with consequent facilitation of the application of [$^3$H]DALECK to characterize isolated opiate receptors and to study the chemical basis of their subtypes.

Acknowledgments

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# Adenosine 5′-Triphosphate at the Active Site Accelerates Binding of Calcium to Calcium Adenosinetriphosphatase†

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ABSTRACT: The complex of Mg·ATP and the calcium adenosinetriphosphatase of sarcoplasmic reticulum ($E$·ATP) reacts with 50–300 $\mu$M $Ca^{2+}$ to form phosphoenzyme ($E$-P·$Ca_2$) with a rate constant of 70 $s^{-1}$ (pH 7.0, 100 mM KCl, 5 mM $MgSO_4$, 25 °C, and SR vesicles passively loaded with $Ca^{2+}$). This rate constant is independent of $Ca^{2+}$ concentration above 50 $\mu$M. It is 4–6 times faster than the rate constants of 11–15 $s^{-1}$ for the conformational change associated with $Ca^{2+}$ binding in the absence of activation by ATP. The reaction of 200 $\mu$M $Ca^{2+}$ with enzyme preincubated in 0.9 $\mu$M [$\gamma$-$^{32}$P]ATP·Mg shows a burst of [$^{32}$P]$E$-P·$Ca_2$ formation. This result indicates that Mg·ATP bound to the active site, and not a regulatory site, can accelerate the conformational change associated with $Ca^{2+}$ binding because this concentration of Mg·ATP is well below the $K_d$ of 160–500 $\mu$M for the putative regulatory site. When an unlabeled ATP chase is added with the $Ca^{2+}$ to enzyme preincubated with [$\gamma$-$^{32}$P]ATP·Mg, the amount of [$^{32}$P]$E$-P·$Ca_2$ that is formed increases with the concentration of ATP in the preincubation solution and is consistent with a maximum fraction trapped of 0.55 and $K_d$ = 4.5 $\mu$M for the dissociation of Mg·ATP from the active site. The fact that labeled $E$·ATP can be trapped by added $Ca^{2+}$ confirms the conclusion that dissociation of ATP from $E$·ATP·$Ca_2$ is slow relative to phosphorylation.

While there is good evidence that ATP accelerates the conformational change associated with $Ca^{2+}$ binding to the calcium adenosinetriphosphatase (Ca-ATPase) of SR[1] (Sumida et al., 1978; Takisawa & Tonomura, 1978; Scofano et al., 1979; Inesi et al., 1980; Guillain et al., 1981; Pickart & Jencks, 1984), there is disagreement as to the mechanism of this effect. One proposal is that binding of ATP at the active site before the conformational change provides an alternate reaction pathway with a faster rate of $Ca^{2+}$ binding (Boyer & Ariki, 1980; Inesi et al., 1980; Pick, 1981). Another possibility is that there is a regulatory site at which ATP acts to increase the rate of the conformational change (de Meis & Boyer, 1978; Scofano et al., 1979; Pick & Bassilian, 1981). Studies of ATP binding at equilibrium have indicated the existence of a second site to which ATP binds with a $K_d$ between 160 and 500 $\mu$M (Yates & Duance, 1976; Dupont, 1977; Nakamura & Tonomura, 1982; Clore et al., 1982). We report here experiments which show directly that ATP bound to the active site accelerates the conformational change associated with $Ca^{2+}$ binding.

## Materials and Methods

*Materials.* Reagents were generally of the highest purity available and were used without further purification. $Na_2$ATP was obtained from Boehringer Mannheim ("Sonderqualitat"), and [$\gamma$-$^{32}$P]ATP (>98% purity) was purchased from New England Nuclear.

Tightly sealed sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a slight modification of the MacLennan procedure, as described previously (Pickart &

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[1] Abbreviations: SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicle(s); $E$, calcium adenosinetriphosphatase; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

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