The pharmacological actions of opiate drugs are best characterized by sedation, analgesia, respiratory depression and changes in temperature. Repeated exposure to opiates, however, produces tolerance to these effects such that the magnitude of response is much reduced or absent. From this it must be assumed that the binding of opiates to their receptor sites activate certain biochemical events resulting in the observed pharmacological responses outlined above, as well as initiating steps leading to tolerance and dependence.

The nature of the biochemical steps producing tolerance is unknown. Earlier proposals (1,2) have been directed toward a study of metabolic adaptation by the cell, however, to date, pharmacokinetic observations of naive and tolerant animals have not accounted for the degree of adaptation to repeated drug exposure nor have they adequately explained physical dependence (3,4,5). A more functional approach attempts to explain tolerance and dependence based on neurochemical adaptations by the cell to repeated opiate exposure.

Theories offered by Shuster (6), Collier (7), and Goldstein and Goldstein (8) define cellular adaptation in terms of enzymes, membrane receptor expansion and feedback mechanisms associated with neurotransmitter synthesis. Collier’s (7) hypothesis suggested that the mechanisms underlying the development of tolerance and dependence involved either a change in the number of receptors and/or their ability to bind opiate ligands. As tolerance develops, opiate agonist sensitivity (reduced) becomes inversely proportional to opiate antagonist sensitivity (increased). It is therefore of interest to view these sensitivities in terms of altered receptor activity, however, several laboratories (9-12) to date have failed to provide evidence for quantitative or qualitative changes in opiate receptors during chronic opiate treatment. Alternatively, one must therefore consider other mechanisms for induction of cellular adaptation leading to tolerance and dependence.

Recent advances in opiate receptor isolation and characterization have prompted a more molecular approach to study of cellular adaptation. The binding of opiate ligands to their respective receptor sites may stimulate membrane receptors which can communicate intracellularly to activate a number of major systems whose cumulative effects generate tolerance. This type of activation process may be similar in nature to the hormonal activation of
membranal adenyl cyclase to increase the intracellular levels of cAMP. However, cellular adaptation to opiates may occur on an even more fundamental level requiring changes in a cellular constituent which serves to integrate broad areas of cellular activity.

### CALCIUM AS A MODULATOR OF CELLULAR FUNCTION

Cell calcium functions in neurotransmitter activity and membrane stability and together with cyclic nucleotides, is thought to play a major role in regulating intracellular metabolism. By virtue of its obligatory involvement with cyclic nucleotides (13,14) and its role in neurotransmitter systems, Phillis (15,16) has suggested Ca++ may function as a primary and secondary messenger in the central nervous system. Table 1 outlines some of the areas of major involvement of Ca++, many of which are also directly or indirectly affected by opiate treatment. Calcium activates tryptophan and tyrosine hydroxylase as well as adenyl and quanyl cyclase and phosphodiesterase activities (17). Calcium is also an obligatory requirement for excitation-contraction (23) and secretion coupling mechanisms (24) and neurotransmitter-receptor interactions (25,26). Its role in membrane stabilization, activation of neurotransmitter release (by functioning as a charge carrier), and preliminary association in the regulation of macromolecule synthesis (27-31) further support a role for cell constituent in cellular adaptation.

### CALCIUM AND OPIATE ACTIONS

**Pharmacological Studies**

Kakunago et al. (33) reported that intracisternal injections of Ca^+^ but not other ions Batt, Met, Sr^++^ or Zntt nor Nat or Kt antagonized opiate induced analgesia. Further EDTA or citrate antagonized Catt’s ability to alter analgesia. This finding was more recently confirmed by Harris et al. (34) who demonstrated that Ca++ antagonism of opiate analgesia is sensitive to EGTA but not EDTA. These investigators also demonstrated that lauthanum, a well-known Ca++ antagonist (35) may have a neuroanatomical site of action similar to morphine in producing analgesia and in fact can substitute for morphine.
in a cross-tolerance situation in producing analgesia (36,37). These later findings support earlier work by Shikimi et al. (38) and his suggestion that the analgesic action of morphine may be due to the opiate's affect on Cat+ flux. While Ca++ was the only cation found to alter analgesia, Mg++ content was observed to increase after acute opiate treatment (39).

Shikimi et al. (40) demonstrated morphine decreased whole brain Ca++ in mice, an effect to which tolerance developed but was independent of analgesia tolerance. In accordance with this finding was the report by Marchand and Denis (41) of increases in urinary excretion of Ca++.

Studies in our laboratory have extended the work of Shikimi et al. (40) by demonstrating opiates in a dose dependent fashion cause Ca++ decreases in the brain in a fairly uniform manner (42). Many hypotheses regarding neurochemical basis for opiate tolerance and dependence regard primary mechanisms in terms of various neurotransmitters which may mediate the tolerance-dependence phenomena. Thus, it would be expected if a particular transmitter were involved, opiate effects may be seen predominately in that brain region where the neurotransmitter distribution is the greatest. Our data based upon assay of Ca++ loss in eight regional brain areas (43) would indicate no predominant area of opiate induced Ca++ loss suggesting a lack of correlation between Ca++ depletion and opiate effects on any transmitter system. Kuhar et al. (44) reported similar lack of correlation between opiate receptor binding and lesioned neurotransmitter regions. Further treatment with maximal Ca++ depleting doses of reserpine (5.0 mg/kg) and morphine (50 mg/kg) demonstrated additive Ca++ depletion indicating that morphine and reserpine sensitive Ca++ pools were in all likelihood distinct. In support of this finding Ross and Lynn (45) and Ross (46) showed reserpine pretreatment had no effect upon development of four hour and seven day tolerance to Ca++ depletion.

The depletion of Ca++ by morphine sulfate was also seen with the opiate congener levorphanol but not with the analgesically inactive (+) isomer dextrorphan. Naloxone effectively blocked the morphine induced decrease in Ca++ but did not prevent reserpine or pentobarbital from reducing calcium levels (47).

Relationship of Ca++ to Development of Tolerance and Dependence
Many factors have been reported to influence the development of tolerance and a dependence on opiate drugs. Among the more prominent are those dealing with use of nucleic acid and protein synthesis inhibitors. Inhibitors of protein synthesis such as cycloheximide and puromycin have been shown to effectively alter development of analgesic tolerance (48). Tolerance to calcium depletion was observed by Shikimi et al. (40) and Ross (46) demonstrated that cycloheximide but not chloramphenicol effectively blocked tolerance to calcium depletion. These studies are summarized in Figure 1. These results together with the lack of effects of reserpine pretreatment suggest two important points. Cycloheximide has been reported to have a locus of action directed to interruption of nerve membrane synthesis (49,50) while chloramphenicol's action is directed toward mitochondrial synthesis. Based upon these findings, rapid tolerance to Ca++ depletion may be explained by changes in synthesis of nerve membrane protein. Secondly, lack of reserpine's effect upon the induction of Ca++ tolerance suggests the locus of action for tolerance development may reside at membrane sites other than those sensitive to neurotransmitter stimulation.

Subcellular Studies: Locus of Ca++ Depletion

The earlier work of Shikimi et al. (40) and previous studies in our laboratory satisfy the criteria necessary to demonstrate calcium depletion as a specific opiate effect. However, it was of interest to us to further explore the locus of this calcium depletion with reference to specific subcellular calcium pools. If the calcium depletion is to be formally considered a specific effect of opiate treatment, one may expect that calcium levels would be altered in those sub-cellular fractions shown to preferentially bind opiate ligands. Pert and Snyder (51) initially reported that 3H-opiate ligands were predominantly bound in vitro to crude mitochondrial fractions (P2) containing nerve endings, with less binding in microsomal (P3) fractions. Pert et al. (52) extended these observations by fractionating the crude mitochondrial fraction over sucrose gradients and demonstrating the majority of opiate binding to be associated with partially purified nerve ending fraction. More recent experiments, administering 3H-opiate ligands in vivo (53,54) have demonstrated association of the ligands with synaptic membrane fractions.

If the calcium decrease is the result of initial binding of the opiate agonist, causing displacement of Ca++, the locus of the calcium decrease may be confirmed by examining Ca++ content in subcellular fractions after acute in vivo treatment. Cardenas and Ross (55) have examined the Ca++ contents in 11 subcellular fractions obtained by Ficol-Sucrose gradients after acute opiate treatment. They report the locus of calcium depletion to be confined to the synaptic particulate fraction. A comparison of the subcellular distribution of opiate ligand binding
Crude separation of the subcellular fractions into P1 (nuclear), P2 (crude mitochondria), P3 (microsomal) and S (soluble) indicate a predominant loss of calcium from the P2 fraction. Similarly, but to a lesser extent, the predominant binding of opiate ligands (52%) occurs in this fraction. Sub-fractionation of the crude mitochondria (containing myelin, synaptic nerve endings and mitochondria) by sucrose (52) or Ficoll-Sucrose (55) reveals the predominant calcium loss occurs in the synaptosome particulate (73%), with a small but significant loss occurring in the myelin (20%). This locus of calcium depletion is similar to the distribution of opiate receptor binding and supports the original premise that the calcium decrease occurs via displacement of synaptosomal Ca++ after opiate binding.

In addition to calcium, magnesium as well plays an important role in function of biological membranes and serves as cofactor for many enzyme systems (56,57). However, Kakunaga et al. (32) were unable to antagonize opiate analgesia with intracisternal injections of magnesium. More recently, it has been demonstrated both Ca++ and Mg++ could inhibit binding of opiate ligands to the crude membrane homogenate (10,51,58). Subsequently, Nat and Mn++ were found to be the most effective in vitro modulators of opiate ligand binding (58-61). While Ca++ was found to be ineffective in differentiating agonist or antagonist binding.

Recent studies in our laboratory have examined the levels of Na+, K+ and Mg++ in subcellular fractions after a single dose of morphine. While calcium depletion was significant and confined to synaptic particulate fractions no changes in Na+, K+ or Mg++ were observed for any of the subcellular fractions (55). It is difficult to resolve at this time whether or not any endogenous cation may be regulating the conformation of a drug receptor. However, Lee et al. (62) have recently suggested, based on comparisons of opiate ligand binding in Tris-HC1 vs. artificial buffers, that the presence of a combination of monovalent and divalent ions best contributes to affinity and accessibility of binding sites.

While the sodium model (58,59) is useful for in vitro discrimination of opiate ligand binding, the presence of 100 mM Nat prevents physiologic uptake/release and binding of Ca++ to synaptic membranes (63-67). An alternative explanation for regulation of opiate ligand binding offered by Cardenas and Ross (55), views the opiate receptor in a Ca++ associated conformation. The
binding of morphine induces Ca++ displacement shifting the membrane to a Ca++-dissociated state which may be reversed by naloxone (47).

**ADAPTATION TO SUBCELLULAR LOSS OF CALCIUM**

As stated earlier, the binding of opiate ligands to their receptor sites is extremely sensitive to inorganic ions (51, 59). Calcium and magnesium, as well as sodium and lithium, but not potassium, have been reported to alter ligand binding (10,58). If cellular adaptation at a membrane level is involved in the induction of tolerance to Ca++ depletion, it would appear that repeated administration of opiates may cause changes in one or more of the monovalent or divalent ions. Figure 2 illustrates the effects of tolerance and dependence upon calcium contents in synaptic particulate material. Twenty-four hours after morphine pellet implant Ca++ levels are significantly lower than control. Following seventy-two hours, calcium levels are significantly increased by 57% over control. The administration of naloxone to morphine pelleted mice reversed the elevated calcium to control levels.

Table 3 outlines the results of similar studies which measured Nat, K+ and Met in synaptosomal particulate from morphine pelleted mice. These studies indicate that Ca++ contents are slightly lowered at twenty-four hours. No changes are reported for Nat, K+ or Met. At seventy-two hours, magnesium was increased 13%. No changes were observed for Nat or K+. Naloxone induced withdrawal response produced a return to control of Met levels while having no effect on the monovalent ions. The slight but significant changes in magnesium observed at seventy-two hours are best described as indirect when compared to changes in particulate calcium, since no changes in Mg++ are seen after acute opiate treatment (thirty minutes) (55) nor twenty-four hours after pellet implant. Pharmacological intervention using Mg++ to inhibit opiate analgesia was also without effect (32,34). These results would suggest that calcium content of the synaptic particulate significantly increases during the chronic exposure to morphine through pellet implant.
This apparent modulation of enzyme activity by the presence or absence of Ca++ may have implications for transport of cations, neurotransmitters and enzyme activities, or more importantly, directly or indirectly affects the activity of nucleotides which are known to play a predominant role in mode of action of narcotics (74). Opiates may also affect the turnover of enzyme activities, transport of neurotransmitters, or possibly neurotransmitter uptake, release, metabolism and degradation by altering enzyme activities. 

In addition, alteration in membrane Ca++ by narcotics may affect the status of Ca++ in cell activation. This may be another mode of action for narcotics (75). Opiates may also affect the Ca++ content in cell membranes. For example, narcotic analgesic administration to rats results in an increase in the level of Ca++ in rat brain homogenate and synaptosomes. Opiates may also affect the Ca++-dependent enzyme activity and Ca++-mobilizing capacity of synaptic membranes. This increase in symptom calcium content may be modulated by opioid antagonists such as naloxone and lanthanum as well as by the binding of narcotics to synaptic membranes. This suggests that narcotic receptors may be involved in the modulation of Ca++ content in cell membranes. 

A further insight into this increase in synaptic calcium content may be obtained from the study of the effects of narcotics on the binding of narcotics to synaptic membranes. The interaction of narcotics with synaptic membranes may be affected by the presence or absence of Ca++. This interaction may be mediated by the binding of narcotics to synaptic membranes in a Ca++-dependent manner. This suggests that narcotic receptors may be involved in the modulation of Ca++ content in cell membranes. 

**TABLE 3**

<table>
<thead>
<tr>
<th>Cation Contents</th>
<th>Control</th>
<th>170 h</th>
<th>17 h</th>
<th>7 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca++</td>
<td>3.20</td>
<td>3.02</td>
<td>2.75</td>
<td>2.46</td>
</tr>
<tr>
<td>Mg++</td>
<td>1.52</td>
<td>1.50</td>
<td>1.43</td>
<td>1.45</td>
</tr>
<tr>
<td>Na++</td>
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<td>5.03</td>
<td>5.05</td>
<td>5.07</td>
</tr>
<tr>
<td>K+</td>
<td>0.30</td>
<td>0.29</td>
<td>0.25</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Significantly different from control.*

The calcium content in synaptic membranes of rat brain homogenate and synaptosomes was significantly increased during chronic narcotic treatment. This increase in calcium content may be modulated by opioid antagonists such as naloxone and lanthanum as well as by the binding of narcotics to synaptic membranes. This suggests that narcotic receptors may be involved in the modulation of calcium content in cell membranes.