Cytochemical Localization of Adenylate Cyclase in Broken Cell Preparations of the Cerebral Cortex

S. W. FRENCH, D. S. PALMER AND M. CALDWELL

SUMMARY: *Broken cell preparations derived from rat cerebral cortical grey matter were studied cytochemically to localize adenylate cyclase (AC) activity in subcellular organelle membranes. AC activity was localized by visualizing reaction product in brain particulate fractions by electron microscopy. Activity was found in the endoplasmic reticulum, on the inside of the inner mitochondrial membrane and on both leaflets of the nuclear membrane. Reaction product was found in the post-synaptic density area of most synapses. The reaction product tended to be more prominent in the presence of flouride. A synaptosome-rich fraction was shown to have NE stimulated AC activity which was blocked in vitro by both a α -and an β -blocker and in vivo by propranolol.*

RÉSUMÉ: *Des préparation de cellules brisées dérivées de la matière grise cortico-cérébrale du rat furent étudiées de façon cytochimique afin de localiser l'activité de l'adénylate cyclase (AC) dans les membranes d'organelles sub-cellulaires. L'activité AC fut localisée en étudiant le produit de la réaction dans les frantions particulaires du cerveau grâce à la microscopie électronique. Nous avons trouvé de l'activité dans le réticulum endoplasmique, à l'intérieur de la membrane mitochondriale interne et sur les deux feuillets de la membrane nucléaire. Le produit de la réaction fut trouvé dans la zone de densité post-synaptique de la plupart des synapses. Ce produit était plus évident en présence de fuoride. Dans un fraction riche en synaptosomes nous avons trouvé une activité AC stimulée par le NE qui était bloquée in vitro par des blockeurs α et β également et in vivo par le propanolol.*

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INTRODUCTION

It is important to determine the sites of adenylate cyclase (AC) activity within the cell. AC is thought to be located in the plasma membrane and its activity stimulated through hormone-receptor interaction (Robison et al., 1970). Logically, AC should face the cell interior and the evidence supported this conclusion (Trams and Lauter, 1974) when HeLa cells, neuroblastoma and fibroblasts were studied. AC may synthesize cAMP at this location for dissemination to the endoplasmic reticulum (ER), to mitochondria where it acts to induce Ca^{2+} efflux from the mitochondria (Borle, 1974) or the nucleus where induction of enzyme synthesis is initiated (Byus and Russell, 1975). There is evidence, however, that AC may be located in the ER (Schulze et al., 1972; and Katz et al., 1974), Golgi (McKeel and Jarett, 1974; Cheng and Farquhar, 1976), mitochondria (Tu and Malhotra, 1973; and Sulimovici and Lunenfeld, 1974) and nuclear membranes (Wedner et al., 1973; and Tu and Malhotra, 1973) as well as the plasma membrane (Schulze et al., 1972; Tu and Malhotra, 1973; Katz et al., 1974; and McKeel and Jarett, 1974) in various cell types. AC at these multiple intracellular sites could facilitiate regulation of cAMP-dependent reactions in the immediate vicinity of AC. It would allow compartmentalization and modulation of cAMP-stimulated events without a lag period that otherwise would result from the time required for cAMP to move from the plasma membrane to the cell interior.

In the present investigation washed homogenates from rat cerebral cortical grey matter slices were

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studied cytochemically for AC localization. Broken cells were used to allow access of chemicals in the medium to AC located within the cell. We were particularly interested in determining if AC was located in the post-synaptic membrane since this is a logical site for the enzyme if cAMP is involved in synaptic transmission as postulated by McAfee et al. (1971).

There is considerable conflicting data in the literature on whether washed particulate fractions derived from brain homogenates have hormone-responsive AC activity. For this reason we have included NE-stimulated cAMP response studies on the washed brain homogenates in order to determine if AC activity does exist in these fractions.

METHODS

Cerebral cortical slices 1mm thick were obtained from four adult male rats under ether anesthesia. The slices were cut parallel to the surface of the brain in the region of the frontal-parietal cortex so as to include as much grey matter and as little white matter as possible.

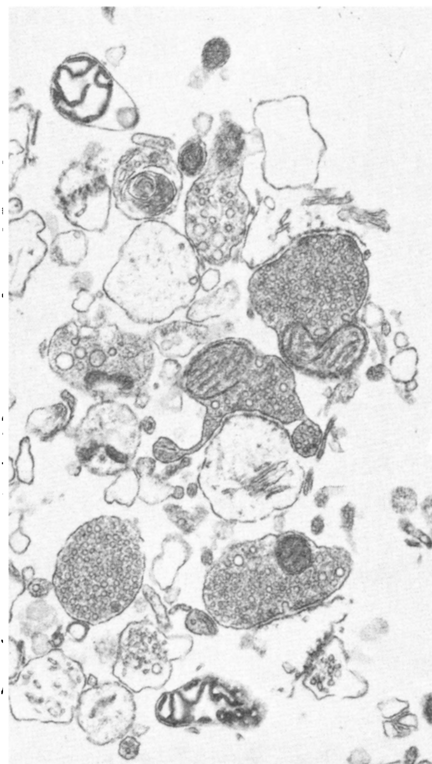

Figure 1—Crude synaptosomal fraction. Note numerous synaptosomes. Free mitochondria are present (38,800x).

For the cytochemical identification of AC active sites the cerebral cortical slices were hand homogenized (12 strokes) with a glass-teflon homogenizer in 10 ml of ice cold solution containing 0.32M sucrose, 5mM EDTA and 5mM Tris buffer pH7.4 (SET). The resulting homogenate (200μl) was added to 2ml of reaction mixture of Sato et al. (1974) and incubated at 30° for 30 min while shaking in air. The incubation medium contained 80mM maleic acid, 80mM Tris base, 220mM sucrose, 2mM theophylline, 2mM $MgSO_4$, and 4mM lead nitrate (pH 7.4). Particulate lead was removed by centrifugation prior to use. Tubes containing the added substrate, 0.5mM adenylyl imidodiphosphate (AMP-PNP) or 0.01M NaF plus substrate, were incubated at the same time. All solutions were prepared just before use. The brain tissue was not fixed prior to assay. The reaction was stopped by transferring the tubes to ice and centrifuging aliquots in microfuge tubes for 3 min (15,000xg). The pellets were fixed with a solution containing 2.5% gluteraldehyde and 01.M sodium cacodylate buffer, pH7.3. The pellets were processed for electron microscopy by the method of French et al., (1972). Sections were not counterstained with uranyl acetate or lead citrate because these stains obscured the AC reaction product. In the case of controls without substrate, stained sections were also examined in order to find the various organelles which might be missed in unstained sections. The sections were examined with a Phillips 300 electròn microscope using 60KV or a RCA 3H using 50kV.

A crude synaptosomal fraction was used to quantitate the AC response to norepinephrine (NE) stimulation. Cerebral cortical grey matter slices from two rats were pooled for each assay. Two experiments were performed. In the first, the synaptosomal fractions were incubated in 10^{-4} and 10^{-5} M NE or with 10^{-4} M NE plus either dl-propranolol or phenoxybenzamine. In the second experiment, the rats were given 10 mg/kg dl-propranolol intraperitoneally and the synaptosomal fractions were obtained and assayed for the AC response 30 min later using 10^{-5} and 10^{-4} M NE.

The crude synaptosomal fractions were prepared after homogenizing the brain slices in SET using 12 strokes in a motor driven glass-teflon homogenizer. The homogenates were centrifuged at 1,000xg for 8 min at 0°. The supernatant was centrifuged at 10,000xg for 10 min. The pellet was resuspended in 40 ml SET and centrifuged for 10 min. at 5,000xg. The fluffy layer was removed and the pellet resuspended in Krebs-Ringer bicarbonate solution to give a protein concentration of 0.15-0.2mg/100 μl. The final pellet was examined for its composition using the method described above (French et al., 1972). The fractions were rich in synaptosomes but scattered myelin, mitochondria and unidentified vesicles were present. Attached fragments of postsynaptic membranes were easily visualised (Fig. 1).

The AC response was quantitated measuring basal levels of cAMP and cAMP levels after incubation with NE for 6 min. The method used was the same as used for brain slices (French and Palmer, 1973). The concentration of the synaptosomal fraction protein used in the assay was 0.075-0.1 mg/ml. Protein was measured by the method of Lowry et al., (1951) and cAMP was determined by the method of Gilman (1970). Assays were done in triplicate and an internal standard was used. The results were analyzed by the Student's "t" test.

RESULTS

Reaction-product was not seen at the plasma membrane of intact brain cells or synaptosomes in any of the rat brain homogenates incubated for AC activity cytochemistry. This was true whether AMP-PNP was present with or without NaF. The only cells to show focal light staining of the plasma membrane were intact smooth muscle cells (Fig. 2) when AMP-PNP was present. Reaction product was also seen in short segments of the myelin membranes. Staining of the postsynaptic density of synaptosomes was obvious in preparations incubated with AMP-

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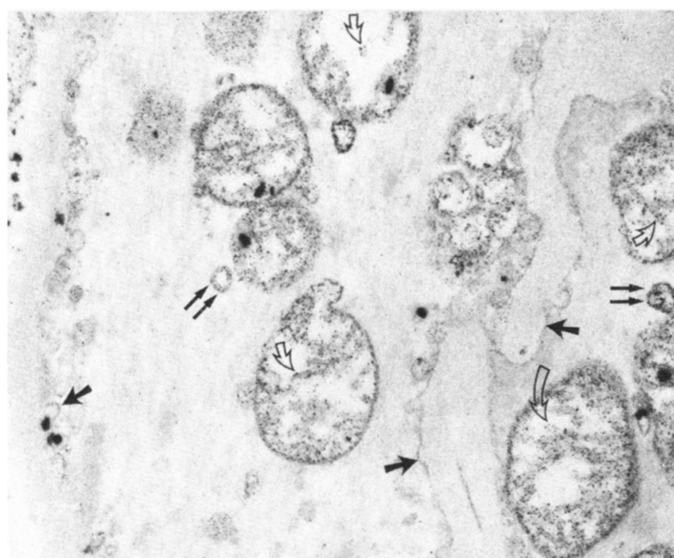

Figure 2—Smooth muscle cell showing AC reaction product in the indentations in the plasma membrane (single solid arrow), ER (double arrows) and on the inner aspect of the inner mitochondrial membrane (open arrows) (59,000x).

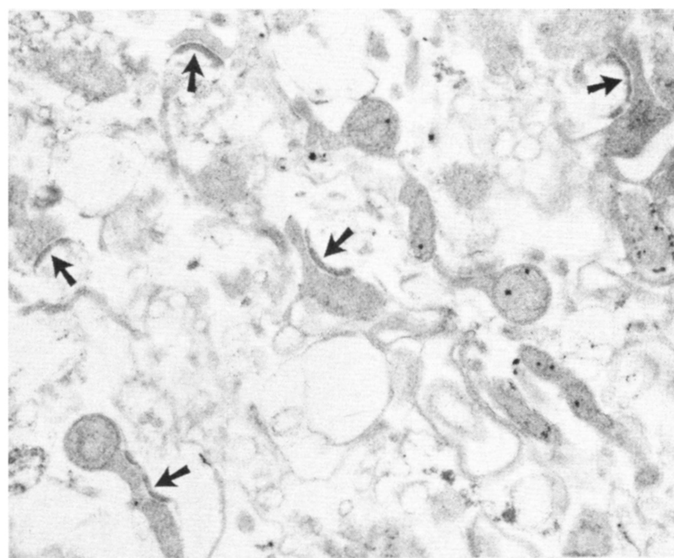

Figure 3—Numerous synaptosomes show AC reaction-product in the post-synaptic density area (solid arrow) (36,000x).

PNP with or without NaF (Fig. 3 and 4). Where the intensity of staining of the postsynaptic density was compared subjectively without knowledge of which photographs were from fluoride treated fractions, it appeared that fluoride increased the reaction product on the average. Some postsynaptic densities did not stain in the presence of substrate. Staining of the post-synaptic membrane with reaction product was absent when no AMP-PNP was added to the incubation medium (Fig. 5).

Certain other cytoplasmic structures of brain cells showed deposits of reaction-product with AMP-PNP whether or not NaF was added to the incubation medium. These structures included mitochondria, endoplasmic reticulum and the nuclear membranes (Fig. 6). These same structures stained in intact smooth muscle cells (Fig. 2). The site of the reaction product in mitochondria was on the inner aspect of the inner membrane for the most part. This is the best seen in smooth muscle cells (Fig. 2) and less consistently in free mitochondria (Fig. 7). Mitochondria within synaptosomes also sometimes contained reaction-product when incubated with AMP-PNP. When the substrate was absent very little reaction product was seen in mitochondria (Fig. 5), although coarse dense deposits were occasionally encountered inside and outside mitochondria (Fig. 5). Occasionally reaction-product was seen between the outer and inner membrane of mitochondria when AMP-PNP was present.

Brain cell nuclear membranes did not invariably show reaction-product in the presence of AMP-PNP (Fig. 8). When the nuclear membranes were separated it could be seen that both inner and outer membranes had deposits of reaction product (Fig. 9). Controls did not show this change. Reaction-product could be seen in the nucleus (Fig. 8) but this was also seen in controls. Reaction-product was also seen in the Golgi apparatus in one cell (Fig. 8). Golgi were not found in control samples. Coarse reaction product deposits were sometimes encountered in large cytoplasmic spaces of controls. These deposits did not resemble the fine deposits seen in samples exposed to substrate.

The quantitative study of AC activity in the crude synaptosomal fractions revealed a significant stimulation of cAMP formation in the presence of 10^{-5} and 10^{-4} M NE ($p < 0.025$) (Fig. 10). Four pair of adult male ad lib. fed rats were used for this study. In the same experiment it was found that propranolol and phenoxybenzamine inhibited the NE-stimulated AC response (Fig. 10). The same assay was repeated using 3 pairs of rats after in vivo propranolol treatment (Fig. 11). Propranolol inhibited the NE stimulated response ($p < 0.05$). Saline injected rats showed a significant response to NE ($p < 0.005$).

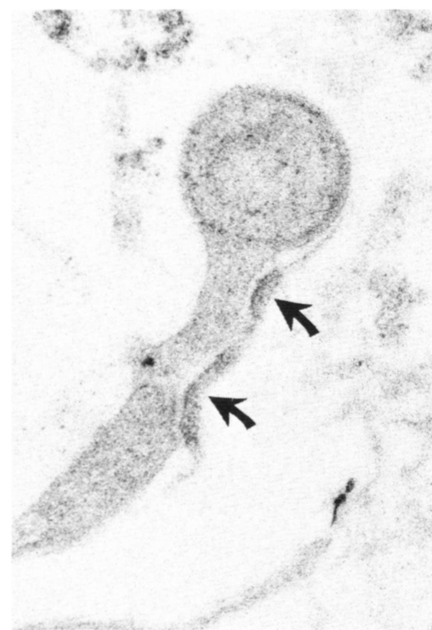

Figure 4—Synaptosome showing reaction-product in the mitochondria and in the postsynaptic density (solid arrow) (68,400x).

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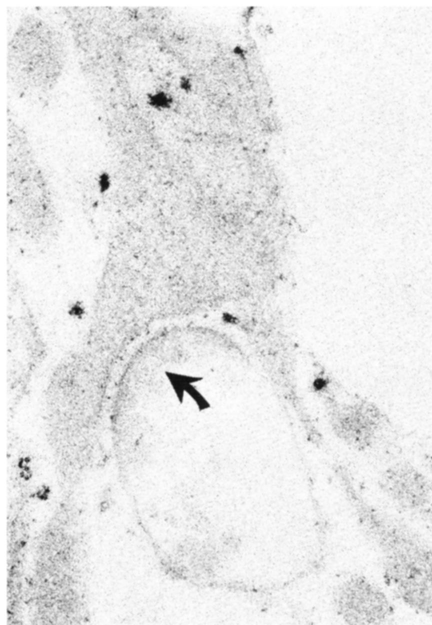

Figure 5—Synaptosome from a homogenate incubated in medium which did not contain substrate (AMP-PNP). There is slight reaction-product in the mitochondria and none at the post-synaptic density (88,500x).

DISCUSSION

The cytochemical evidence reported here supports the conclusion that AC is located at the membranes of the ER the inner aspect of the mitochondrial cristae and the inner and outer nuclear membrane. Of specific interest to the nervous system was the finding that AC was located in the post-synaptic density rather than in the synaptic membranes. This post-synaptic location was first illustrated by Joó and Tóth (1975) and later confirmed by Rechardt and Härköen (1977). AC, therefore, may occupy the same site, post-synaptically, as does cyclic 3',5'-nucleotide phosphodiesterase in the cerebral cortex (Florendo et al., 1971). Functionally, co-existence of the two enzymes at this site would facilitate modulation of cAMP levels at this location. This observation adds strength to the hypothesis that cAMP mediates the chemical transmission by neurotransmitters at the post synaptic membrane (Ueda et al., 1973). Several studies have shown that synaptic transmission increases post-synaptic cAMP (McAfee et al., 1971; McAfee and Greengard, 1972 and Kebabian et al., 1975).

It was of interest to find AC activity on the inner aspect of the mitochondrial cristae and on the inner aspect of the inner membrane. If confirmed by others, this is an important observation because cAMP-dependent protein kinase is latent in mitochondria (Kleitke et al., 1976). The possibility remains that the reaction-product resulted from the ATPase located at this site. Two facts are against this possibility. First, AMP-PNP, the substrate used in the assay, is a potent competitive inhibiter of mitochondrial ATPase (Penefsky, 1974: and Melnick et al., 1974). Second, fluoride inhibits ATPase but fluoride did not inhibit the AMP-PNP supported deposit of reaction-product in our study. Drummond and Duncan (1970) found that 8mM fluoride inhibited 67% of the ATPase activity of heart muscle.

The question then arises, why did we observe AC activity in the mitochondria and nuclear membrane in the brain homogenates when others using other tissues have only seen activity in the plasma membrane and ER? The answer may be that we used broken cell preparations whereas others have used intact cells. The studies of Tu and Malhotra (1973) support this explanation. They found that fungal spores that had been sectioned showed AC localization in mitochondria (between the inner and outer membranes) and nuclear membranes (between the two membranes), whereas intact spores did not show any dense reaction pro-

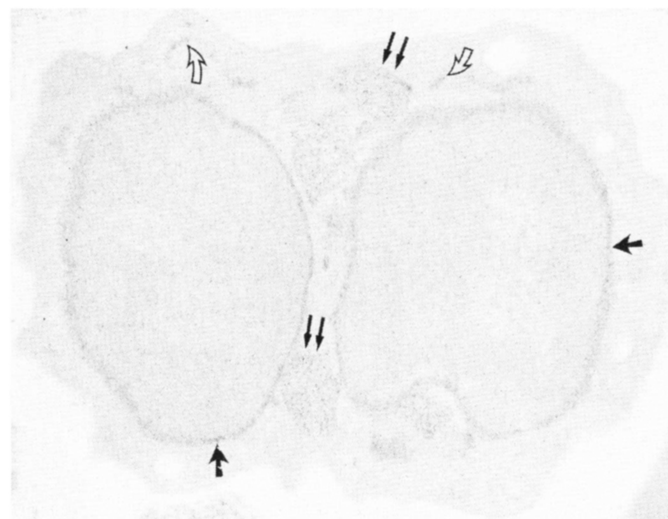

Figure 6—Cell from the brain homogenate showing the reaction-product in the nucleus, nuclear membrane (solid arrow), ER (open arrow and mitochondria (double arrows) (43,000x).

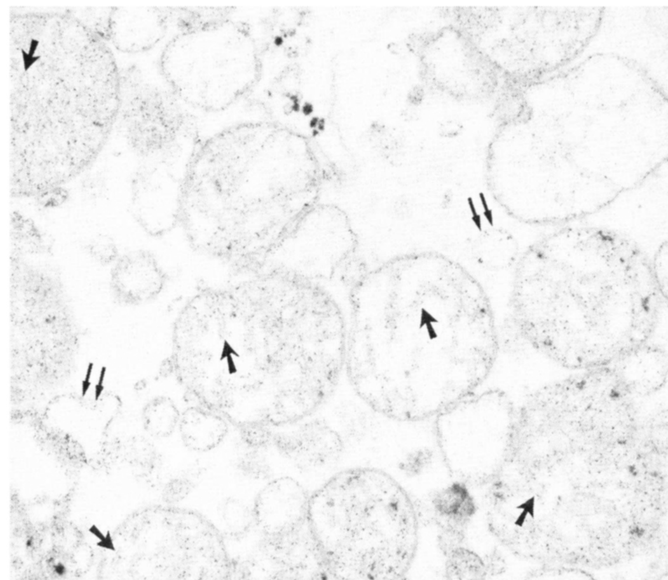

Figure 7—Reaction-product in free mitochondria (single arrow) and microsomal vesicles (double arrows). Note the reaction product on the inner aspect of the cristae (59,000x).

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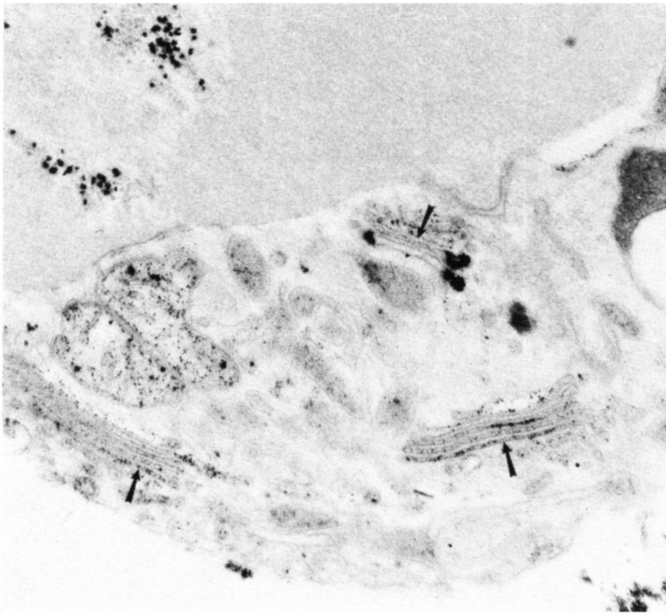

Figure 8—Cell from the brain homogenate showing reaction product in the Golgi apparatus (solid arrows) (73,000x).

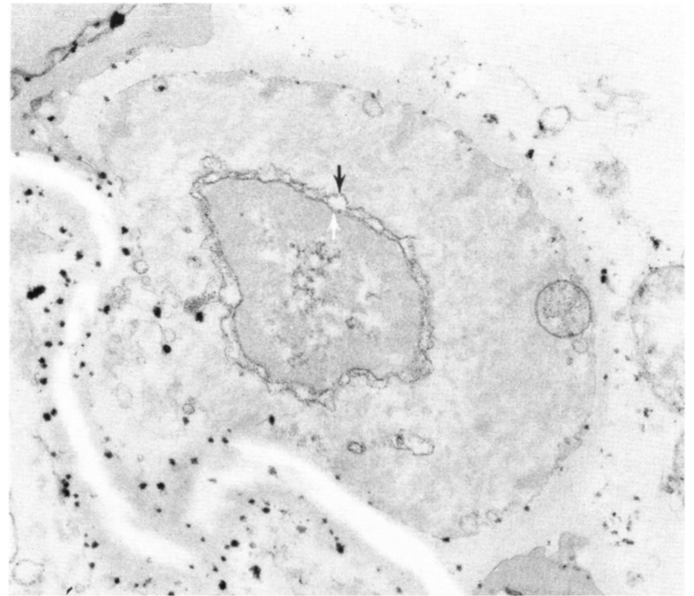

Figure 9—Smooth muscle cell showing reaction product on the outer (black arrow) and inner (white arrow) membrane of the nuclear envelop (39,000x).

duct. They postulated that the thick cell wall of the spores imposed a barrier to penetration by some of the constituents in the incubation media. Some of the smooth muscle cells observed in our broken brain cell preparation that showed nuclear and mitochondrial staining had intact plasma membranes. This suggests that a second factor may account for the staining for AC in the mitochondria and nuclear membranes observed by us. There are two differences in methodology which could account for our observations. First, AMP-PNP was used as substrate rather than ATP. Second, the brain homogenates were not fixed in gluteraldehyde prior to the enzyme assay. AMP-PNP is more specific for AC than is ATP (Howell and Whitfield, 1972). Also, it is possible that prior fixation selectively inhibits AC at certain intracellular sites. The distribution of reaction product in the mitochondria and nuclear membranes in the brain cells was quite different than observed in sectioned fungal spores (Tu and Malhotra, 1973).

The finding of AC activity by cytochemical means in the ER, Golgi, mitochondria and nuclear membranes and in the post-synaptic density makes interpretation of quantitative data derived from brain tissue slices and broken cell preparations difficult. It may be that hormone-stimulated AC activity reflects more than just the response of the post-synaptic membrane receptors to agonists. The AC response of the crude synaptosomal fractions to NE reported here could involve receptors located in the mitochondria or elsewhere. The problem of locating the NE receptor binding sites on cell membranes is presently controversial. (Cuatrecasas et al., 1974; Venter and Kaplam, 1974; and Yong and Richardson, 1974). Others, (Sulimovici and Lunenfeld, 1974; Sulimovici, et al., 1975; French et al., 1976), have found AC activity in mitochondria and other intracellular organelles which was responsive to hormone and NaF stimulation (Katz, et al., 1974; Sulimovici, et al., 1975). The results are consistent with the hypothesis that intracellular organelles have their own receptor-AC mediated response to agonist which allows hormone modulated responses in localized intracellular compartments, independent of the plasma membrane.

Some investigators have found that broken cell preparations derived from brain fail to respond significantly and reproducibly to neurohormones including NE. The problem has recently been reviewed by Drummond and Ma (1973). De Belleroche et al., (1974) most recently reported that the rat brain synaptosomal fraction AC did not respond to NE. On the other hand, Von Hungen and Roberts (1973) have reported that NE consistently stimulated AC activity in cell-free preparations from the rat cerebral cortex but stimulation did not exceed 20% unless EGTA was added to the medium. These authors reported that propranolol blocked the NE stimulated AC response but, unlike our data, they found no blocking with phenoxybenzamine. They did find that the α-blocker, haloperidol, did inhibit the NE stimulated AC response of the brain particulate fraction.

The fact that propranolol administered in vivo blocked the cAMP response of the isolated synaptosomal fractions to NE probably has functional significance since propranolol alters behaviour in animals and man (Orzack et al. 1973; Yorkston et al., 1974; and Navarro et al., 1976). Propranolol is concentrated in the brain as well as lung, liver kidney

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and heart (Hayes and Cooper, 1971), indicating that it probably passes the blood-brain barrier and binds to receptors located in the washed synaptosomal fraction.

The validity of the lead nitrate method for the histochemical demonstration of AC activity in tissues has been questioned (LeMay and Jarett, 1975) on the grounds that lead inhibits AC of pancreatic islet homogenates and fat pad capillaries and that lead hydrolyzes the substrate nonenzymatically. However, Cheng and Farquhar (1976) did not confirm this using liver Golgi and microsomal fractions. They reported that lead inhibited but did not eliminate AC activity possibly because EGTA preserved AC activity during the isolation procedure. It should be pointed out that EDTA which has chelating properties similar to EGTA was utilized in our study but neither chelator was used by LeMay and Jarett (1975). Unlike Cheng and Farquhar (1976), we found the reaction product to be localized to the lumen side of the Golgi. Like Cheng and Farquhar (1976), we found the bulk of the reaction product in the dilated ends of the cisternae (Fig 8).

Two problems remain in interpreting our results. First, we use 4mM lead as the trapping agent for demonstrating AC, a concentration which Lemay and Jarett (1975) found completely inhibited AC. Cutler (1975), on the other hand, found AC activity measured biochemically in a variety of tissues persisted despite the presence of 4mM lead and gluteraldehyde fixation. Indeed, Cutler (1975) showed that the remaining AC activity was stimulated by fluoride and PGE_1. They showed that equivalent cytochemical localization of reaction product occurred in the assayed tissues. The second problem posed by Lemay and Jarett (1975) is that the activity of AC is too low to produce enough PNP from the substrate to exceed the solubility product so that no precipitate would be formed to mark the site of AC cytochemically. This argument, however, ignores the basic principal of cytochemistry which is that the reaction product is much more concentrated at the location of the enzyme than is reflected by the concentration of the reaction product measured in the whole homogenate.

In summary, there are several reasons why we believe that the cytochemical localization of AC in the postsynaptic density is valid. One is that the reaction product localizes to the post synaptic density whether AMP-PNP or ATP is used as substrate (Rechardt and Härkönen, personal communications) and whether lead or cobalt is used as the trapping agent (Joó and Tóth, 1975 and Rechardt and Härkönen, 1977). Since cobalt stimulates AC activity and localizes AC at the same site as lead does, it is unlikely that the inhibition of AC by lead interferes with the cytochemical localization of AC qualitatively. Another reason is that floride seemed to increase the reaction product found in the postsynaptic location in our experiments. This is contrary to the results of Cheng and Farquhar (1976)

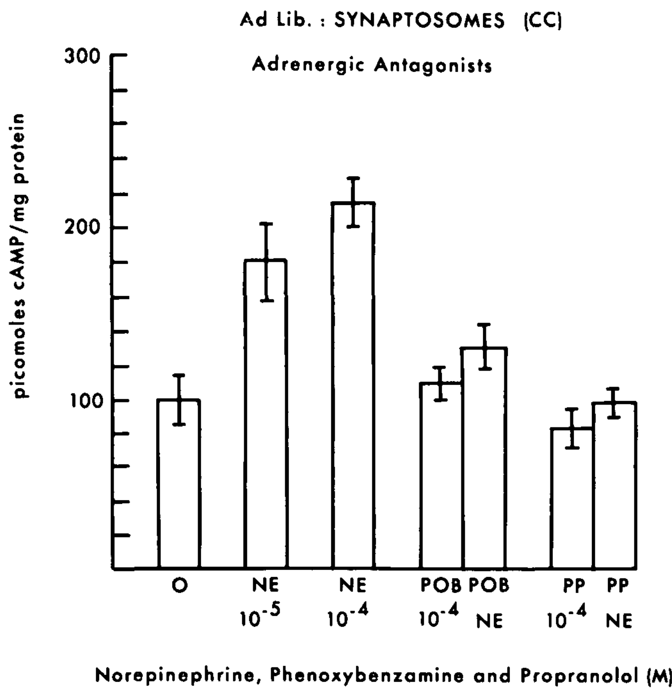

Figure 10—AC response of the cerebral cortical crude synaptosomal fractions to NE and NE plus antagonists. The data is from four pair of rats, except the antagonist data which is from three pair of rats.

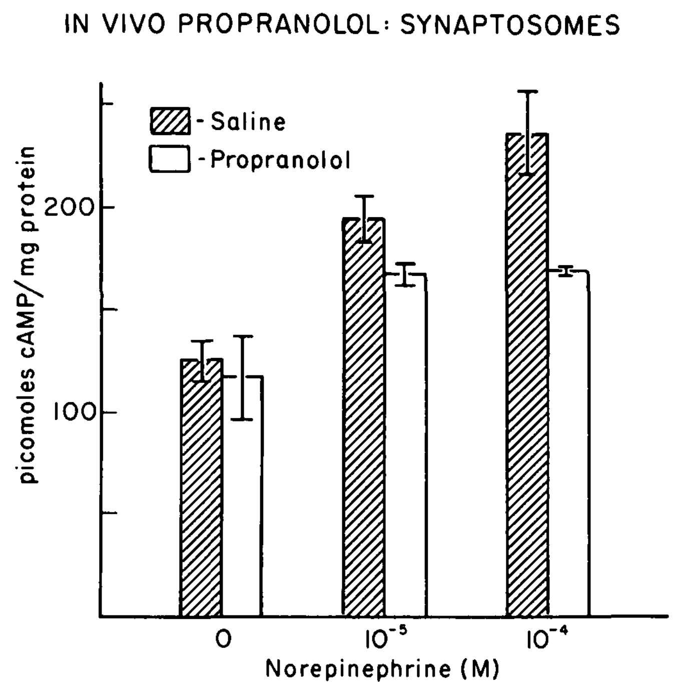

Figure 11—Effect of in vivo propranolol on the AC response of the synaptosomal fraction to NE. The data is from three pair of rats.

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who found no increase in AC in the presence of fluoride biochemically or cytochemically when isolated Golgi were studied. Lastly, we inhibited ATPase with fluoride and Rechardt and Härkönen (1977) inhibited ATPase by altering the pH. We, therefore, consider it unlikely that the reaction product formed at the post-synaptic density was due to ATPase activity.

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