AN *IN VITRO* STUDY OF THE IONOPHORE PROPERTIES OF THE ACETYLCHOLINE RECEPTOR IN *TORPEDO CALIFORNICA* MEMBRANE FRAGMENTS

Thesis by

Donald Miller

In Partial Fulfillment of the Requirements
for the Degree of
Master of Science

California Institute of Technology

Pasadena, California

1976

(Submitted June 1, 1976)

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael A. Raftery for his patient support and guidance throughout this project.

I would also like to thank John Racs for providing many of the preps used in this project. Last, but not least, I would like to thank Terry Moody, Carol Linden, and Frank Stackhouse for their enduring moral support.

ABSTRACT

An *in vitro* system for studying the ionophore properties of the nicotinic acetylcholine receptor from the electric organs of *Torpedo californica* was developed. Efflux of $^{22}Na^{+}$ from microsacs rich in AcChR was studied as a function of the addition of various agonists and antagonists to the medium. In the presence of agonists increased rates of efflux of $^{22}Na^{+}$ occurred, and this affect was abolished in the presence of antagonists. The *in vitro* response to varying concentrations of carbamylcholine was determined. The desensitization phenomenon occurring *in vivo* was also found to occur *in vitro*.

TABLE OF CONTENTS

INTRODUCTION

The acetylcholine receptor (AChR) may be defined as that entity which mediates an initial change in the permeability pf the postsynaptic membrane to certain cations upon the binding of the neurotransmitter acetylcholine (ACh). Most physiological and pharmacological studies on the AChR have been done on the vertebrate neuromuscular junction and on the electroplaque of the electric eel, Electrophorus electricus (1-3). Most biochemical studies of the AChR have been undertaken on preparations isolated from electric organs of electric fish. These organs have been found to contain high concentrations of AchR (4-6). E. electricus, Torpedo marmorata, and T. californica (marine electric rays) have been most widely utilized.

A great deal is known about the biochemistry of the AChR from electric fish. The AChR has been isolated and purified from T. californica and shown to be a glycoprotein with an approximate molecular weight of 380,000 daltons (7). Sodium dodecylsulfate electrophoresis work has indicated there are four subunits of this AChR and their molecular weights have been determined (41, 51, 60 and 54 thousand daltons). Antibodies against the purified subunits have been isolated (8). Ligand-binding studies have shown there are two cholinergic binding sites, one of high (K_D = 2 nM) and one of low

(K_D = 20 nM) affinity, per AChR (9). Interconversion between varying states of affinity of the AChR for ligands has also been studied (10,11).

Although much biochemical work has been done on AChR isolated from T. californica there has been no in vitro measurements of permeability changes in AChR-rich membrane fragments from this animal. It is essential to know in detail the effects of ligand binding and other changes in the AChR environment on its action as an ionophore. Most experiments studying AChR-induced permeability changes of membranes have been in vivo electrophysiological experiments utilizing either the vertebrate neuromuscular junction or isolated whole electroplaques from the electric organ of E. electricus. Such studies are not feasible with Torpedo, since the electroplaque cells are thin and firmly embedded in connective tissue. Therefore, a system was developed whereby AChR-induced permeability changes to Na^+ cations can be observed by monitoring efflux of $^{22}Na^+$ from membrane fragments prepared from crude homogenates of T. californica electric organs. These membrane fragments formed closed vesicles (12) and retain their excitabilty in vitro, i.e. upon addition of cholinergic agonists an increased permeabilty to $^{22}Na^+$ occurs. Thus an in vitro system exists for studying the ionophore activity of the AChR in which parameters in the environment can be carefully controlled. Other workers have developed similar systems for studying electric eel AChR (13,14), but this is the first

time the ionophore activity of the AChR has been studied in T. californica membrane fragments. As the results will demonstrate, this system gives data that correlate closely with those one would expect from previos in vivo studies on the AChR. The studies on ligand binding together with this in vitro system for studying permeabilty changes in AChR-rich membranes should yield valuable information about the molecular events leading to postsynaptic depolarization.

EXPERIMENTAL

AChR-rich crude membrane fragments were prepared from the electric organs which either had been stored at -90°C or were dissected from just-killed T. californica. The organs were then cut up to pieces approximately one inch square and suspended in a volume of cold Torpedo Ringer's solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% N_3^-, 5 mM Tris, pH 7.4) equal to half the weight of the organ, containing a few crystals of phenylmethyl sulfonyl-fluoride to prevent proteolysis and a very small amount of 2,6-di-tert-butyl-4-hydroxymethyl phenol to prevent oxidation. These were then homogenized in a Waring laboratory blendor for 2.5 minutes at top speed under argon. The homogenate was then centrifuged at 4,080 g for 10 minutes in a GSA-rotor of a Sorvall centrifuge. The supernatant was collected, passed through two layers of cheesecloth, and centrifuged at high speed (90,000 g) in a Beckman type 35 rotor in a Beckman L3-50 ultracentrifuge for 1 hr. The supernatant of the high-speed spin was discarded and the pellet resuspended in a volume of Torpedo Ringer's equal to one eighth the weight of the original organ, using a Virtis apparatus at full speed for 45 seconds.

This amount of receptor in the resuspension was determined by the DEAE-paper disk method of Schmidt and Raftery (15) using ^{125}I-α-Bgt, a constituent of the venom from the

krait Bungaris multicinctus which binds specifically and irreversibly to the AChR. Concentrations of receptor in the resuspension averaged around 30 μg ^{125}I-α-Bgt bound per ml of solution, or about 4 μM in toxin binding sites.

Measurements of the permeability of the microsacs to $^{22}Na^{+}$ were initially routinely performed in the following manner:to 1 ml of membrane suspension was added 100μl of an aqueous solution of ^{22}NaCl (Radiochemical Centre, Amsesham, Great Britain) containing 0.2 mC/ml of $^{22}Na^{+}$. The suspension was then stored overnight at 4°C to allow equilibration of the $^{22}Na^{+}$ inside and outside the microsacs. The flux assay was started by the 20-fold dilution of the radioactive suspension into nonradioactive Torpedo Ringer's with or without a cholinergic effector(s). At desired time intervals 0.5 ml aliquots were adsorbed onto tightly packed DEAE columns and washed in a method previously developed in our laboratory (16). $^{22}Na^{+}$ counts were then eluted from the columns by washing with 3 mls Triton X-100 in 10 mM Tris, pH 7.4 which solubilizes the microsacs. Collected efluents were then dried and counted in a dioxane-based scintillation fluid.

Using the above method I was able to take aliquots only as rapidly as one per 1.5 minutes. It was decided this method was undesirable for observing fast processes going on in the system and also was undesirable becuase the precision of the results was less than desirable. I therefore undertook the

the development of a system utilizing Millipore filters for trapping of the microsacs. The system finally arrived at was one utilizing a 12-place Millipore manifold (XX2500) onto which twelve filters could be placed simultaneously. Various pore size millipore filters were tested for scatter in the data and rapidity of wash rates. Two 0.8 μ (AAWP 02500, 0.8 μ , white plain, 25 mm) filters per sampling was decided to give the most favorable results. Applying 0.2 mls of radioactive suspension to each pair of filters and washing with 2 x 7.5 mls of non-radioactivity increased my time resolution to 10 seconds per sampling. The precision of the data was also increased.

RESULTS

Pharamcological Studies

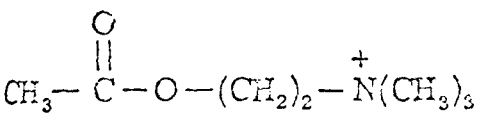
If this *in vitro* system is to be considered valid, it is necessary to compare its response to various ligands with the responses known from *in vivo* studies. Therefore, the rates of efflux of $^{22}Na^{+}$ from the microsacs were studied as a function of the addition of various effectors whose effect *in vivo* is known. Some of these effectors are shown in Fig. 1. Acetylcholine (ACh) and its structural analog, carbamylcholine (Carb), are agonists of the AcChR (17), while d-tubucurarine (dTC) and α-bungarotoxin (α-Bgt), are antagonists (18 - 20). Therefore, one would expect increased rates of $^{22}Na^{+}$ efflux from microsacs when carb on ACh are present, and one would expect varying degrees of blockage of this effect when dTC or α-Bgt are present in addition to the agonists. Fig. 2 shows an example of increased ion flux in the presence of 50 μM carb and the reversal of this effect by 100μM dTC. Fig. 3 shows increased ion flux in the presence of carb and to blockage by pre-incubating the microsacs with α-Bgt prior to addition of carb.

Histrionocotoxin (Htx), isolated from the Columbian arrow-poison frog *Dendrobates histrionicus*, has been shown to decrease the ionic conductance increase caused by ACh in

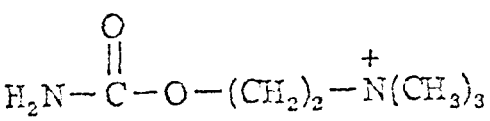
FIGURE 1

Structures of the cholinergic effectors studied *in vitro* on *Torpedo californica* microsacs. They are classified here according to their believed modes of action.

Agonists

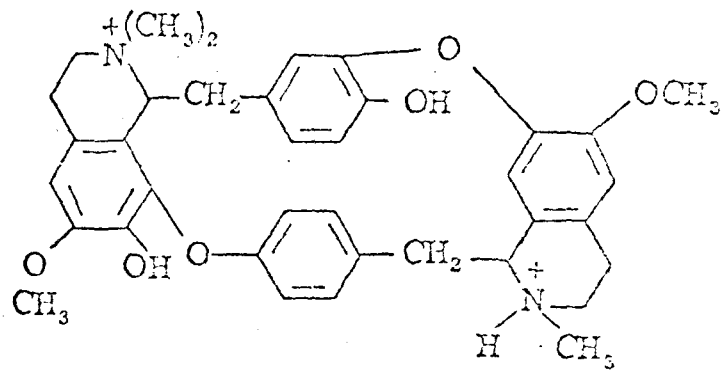


Acetylcholine



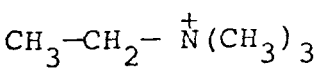
Carbamylcholine

Antagonists



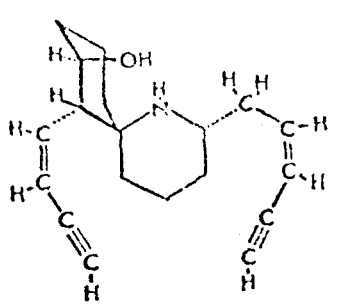
d-Tubocurarine

α – Bungarotxin



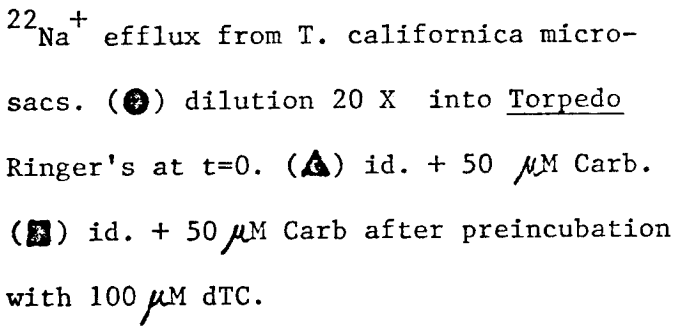
Choline

Local Anaesthetic?



Histrionocotoxin

FIGURE 2



$^{22}Na^{+}$ efflux from T. californica micro-sacs. (●) dilution 20 X into Torpedo Ringer's at t=0. (▲) id. + 50 μM Carb. (■) id. + 50 μM Carb after preincubation with 100 μM dTC.

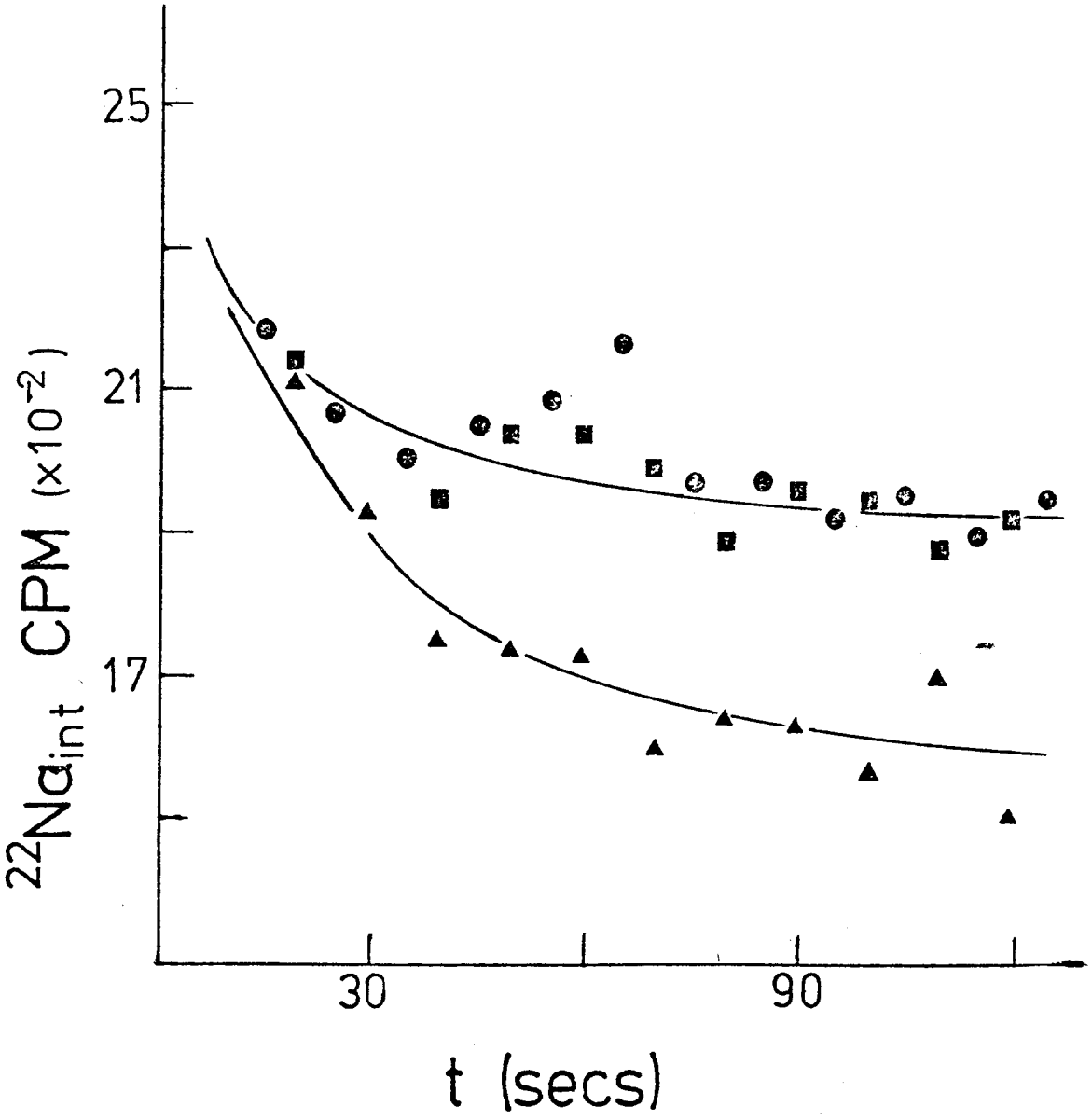
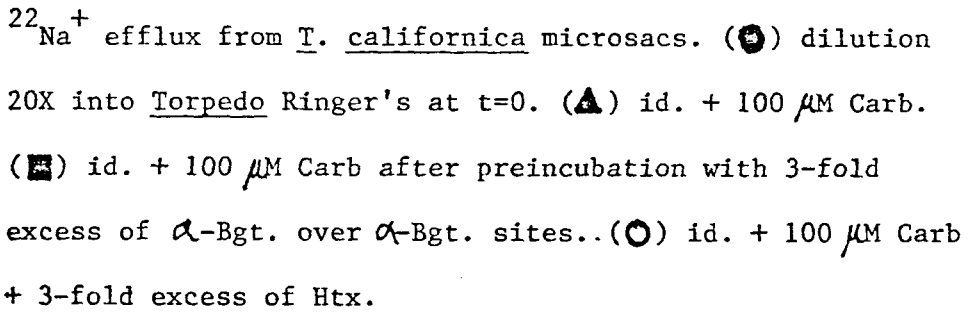
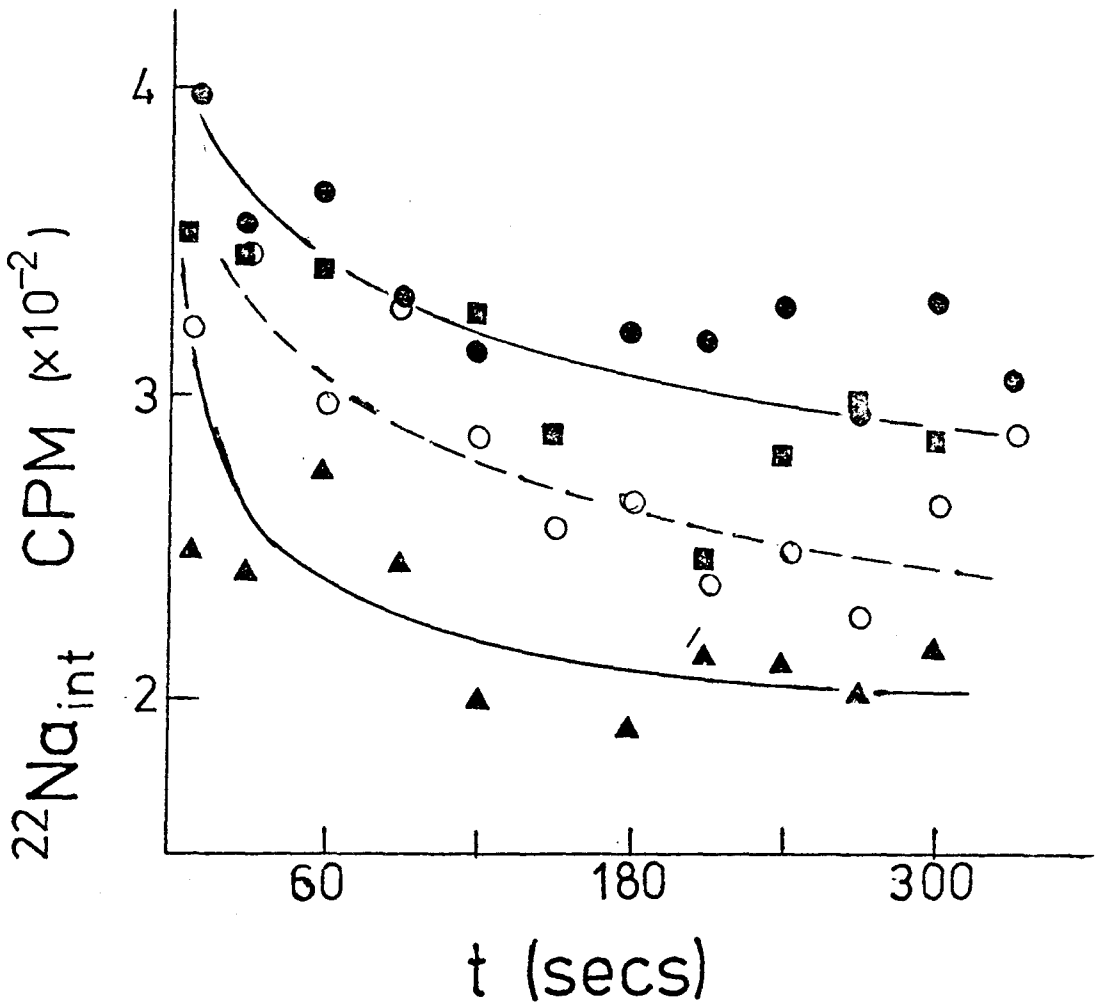

25
21
17
$^{22}Na_{int}$ CPM ($\times 10^{-2}$)
30
90
t (secs)

FIGURE 3



$^{22}Na^{+}$ efflux from T. californica microsacs. (●) dilution 20X into Torpedo Ringer's at t=0. (▲) id. + 100 μM Carb. (■) id. + 100 μM Carb after preincubation with 3-fold excess of α-Bgt. over α-Bgt. sites..(○) id. + 100 μM Carb + 3-fold excess of Htx.


4
3
2
$^{22}Na_{int}$ CPM ($\times 10^{-2}$)
60
180
300
t (secs)

eel electroplaques (21) and the vertebrate neuromuscular junction (22). Fig. 3 shows the similar affect of Htx in the T. californica flux system.

In vivo studies have shown that choline does not act as an agonist (23). Figure 4 shows that in vitro it also does not act an as agonist.

In analyzing some of the initial experiments it was found that substracting the control data from the excited data gave an efflux rate describable as a single exponential function. Thus if the rate of efflux of ^{22}Na in the absence of agonist in some series of exponentials:

$$[^{22}Na]_t = [^{22}Na]_{t=o} (a_1 e^{-k_1 t} + a_2 e^{-k_2 t} + \ldots a_n e^{-k_n t}) \quad (1)$$

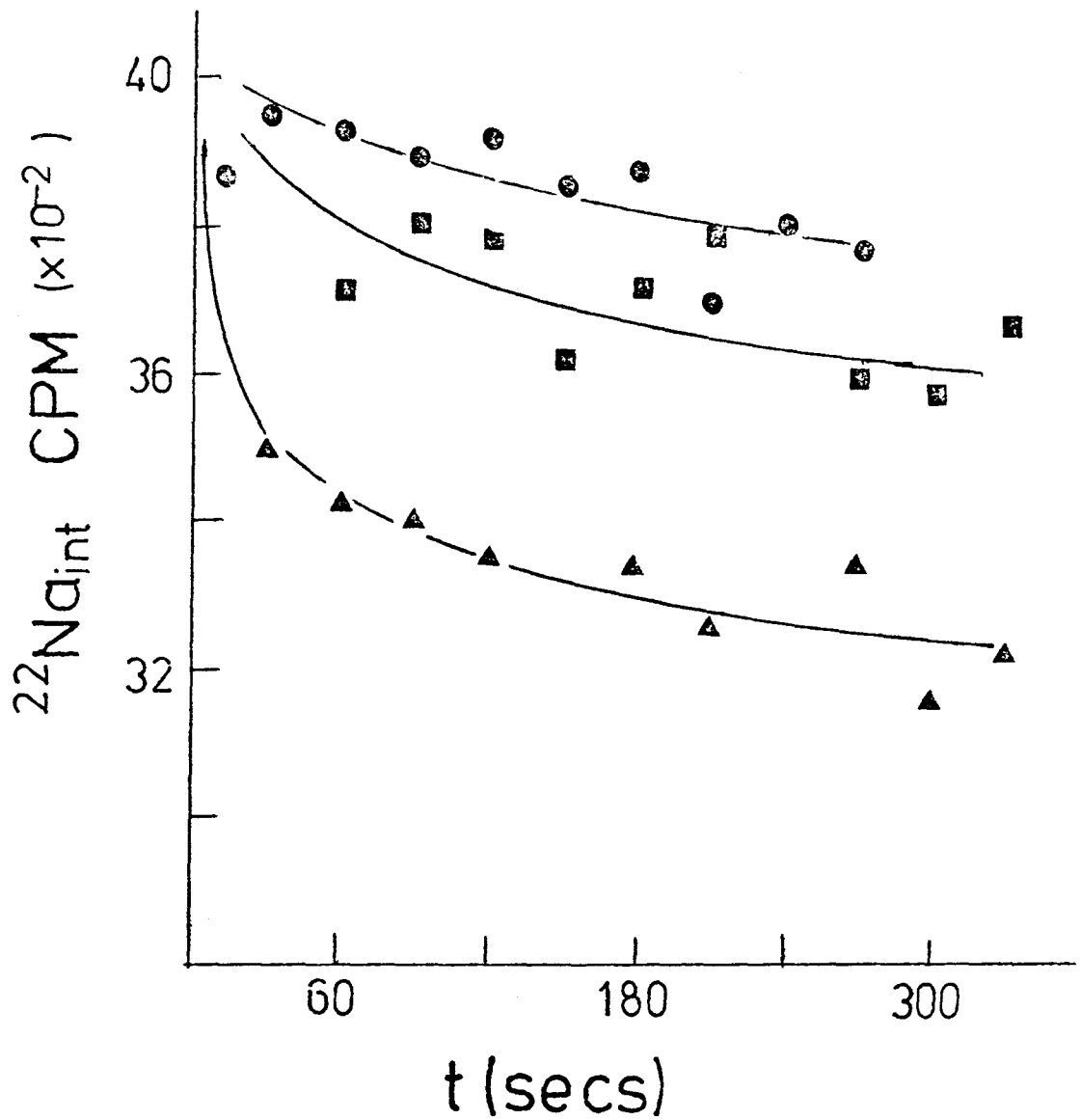
where $[^{22}Na]_t$ = concentration of ^{22}Na inside the microsacs of a given time t, then the rate of efflux in the presence of agonists would then be

$$[^{22}Na]_t = (1) + [^{22}Na]_{t=0} (a_{n+1} e^{-k_{n+1} t}).$$

In the control data we do not see this extra term for as long as 30 min. after that the initial 20 x dilution, and this term is manifested only by the presence of agonist during this time. This implies that the fraction of excitable microsacs are intrinsically less permeable in the absence of agonists than are the non-excitable microsacs. To test this, experiments were done in which the radioactive microsac suspension was diluted 20 x into non-radioactive buffer, and

FIGURE 4


$^{22}Na^{+}$ efflux from T. californica microsacs. (●)dilution 20X into Torpedo Ringer's at t=0. (▲)id. + 100 μM Carb. (■) id. + 100 μM choline.


40
36
32
$^{22}Na_{int}$ CPM ($\times 10^{-2}$)
60
180
300
t (secs)

several hours lapsed (usually 2.5) before addition of small volumes of agonists and subsequent sampling of the suspension. In this manner it was possible to observe receptor-mediated ion flux without the data being obsured by the flux from the non-excitable components of the preparation. Other workers using microsacs from Electrophorus electricus have found similar effects (Hess et al.) Fig. 5 shows an experiment done in this fashion showing excitability by Carb and blocking of this effect by dTC. Note the increased resolution between the control and the excitability data.

Dose-Response Curve

In order to quantitate the response of the microsacs to Carb, the excitability induced by varying concentrations of Carb was measured. Fig. 6 shows the in vitro dose-response curve obtained. The excitability is expressed as the percentage of the maximim excitability obtained for a given preparation. Excitability was defined as the percentage of the Control CPM at 60 seconds elicited by each concentration of Carb.

The midpoint of the dose-response curve, or the apparent "K_D", is approximately 50 μM. The shape of the curve is sigmoid, and is converted to a straight line in the double logarithmic plot of Hill (24). The slope of the line (or Hill coefficient) is].5, indicating apparent cooperativity.

FIGURE 5

$^{22}Na^{+}$ efflux from T. californica microsacs. 2.5 hours after diluting 20X into Ringer's. (●) Control, i.e. no addition of effectors. (▲)Carb added 2.5 hours after dilution such that the Carb concentration is 50 μM. (■) id. with prior addition of dTC such that the concentration of dTC is 100 μM.

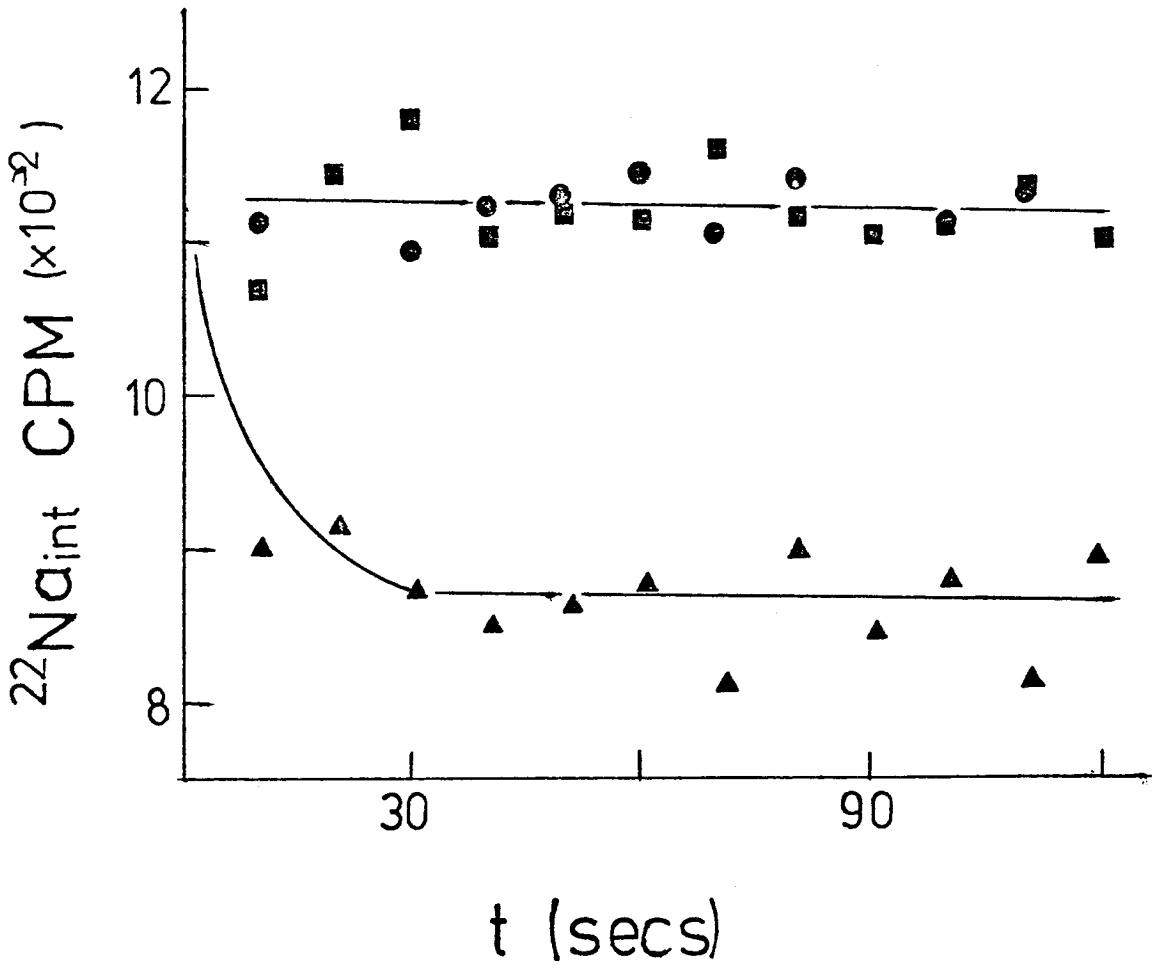
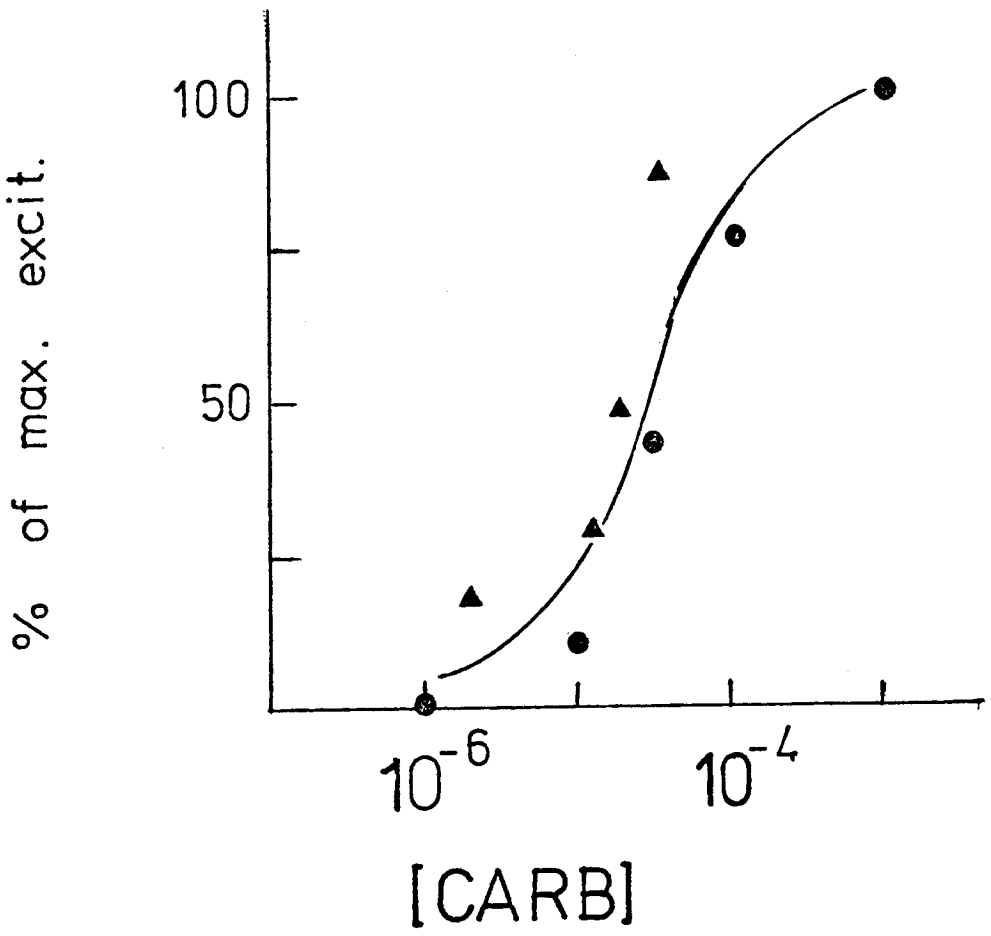

12
10
8
$^{22}Na_{int}$ CPM ($\times 10^{-2}$)
30
90
t (secs)

FIGURE 6

Dose-response curve of the Torpedo microsacs to Carb. The excitability is expressed as a percentage of the maximum excitability for a given preparation. (●) and (▲) represent values obtained from two different preparations.

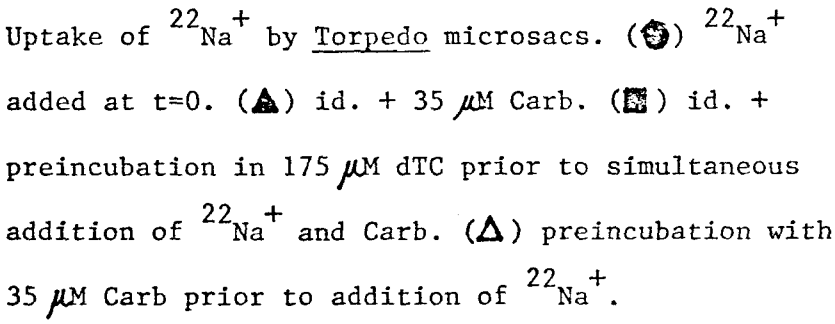

% of max. excit.
100
50
10^{-6}
10^{-4}
[CARB]

Desensitization

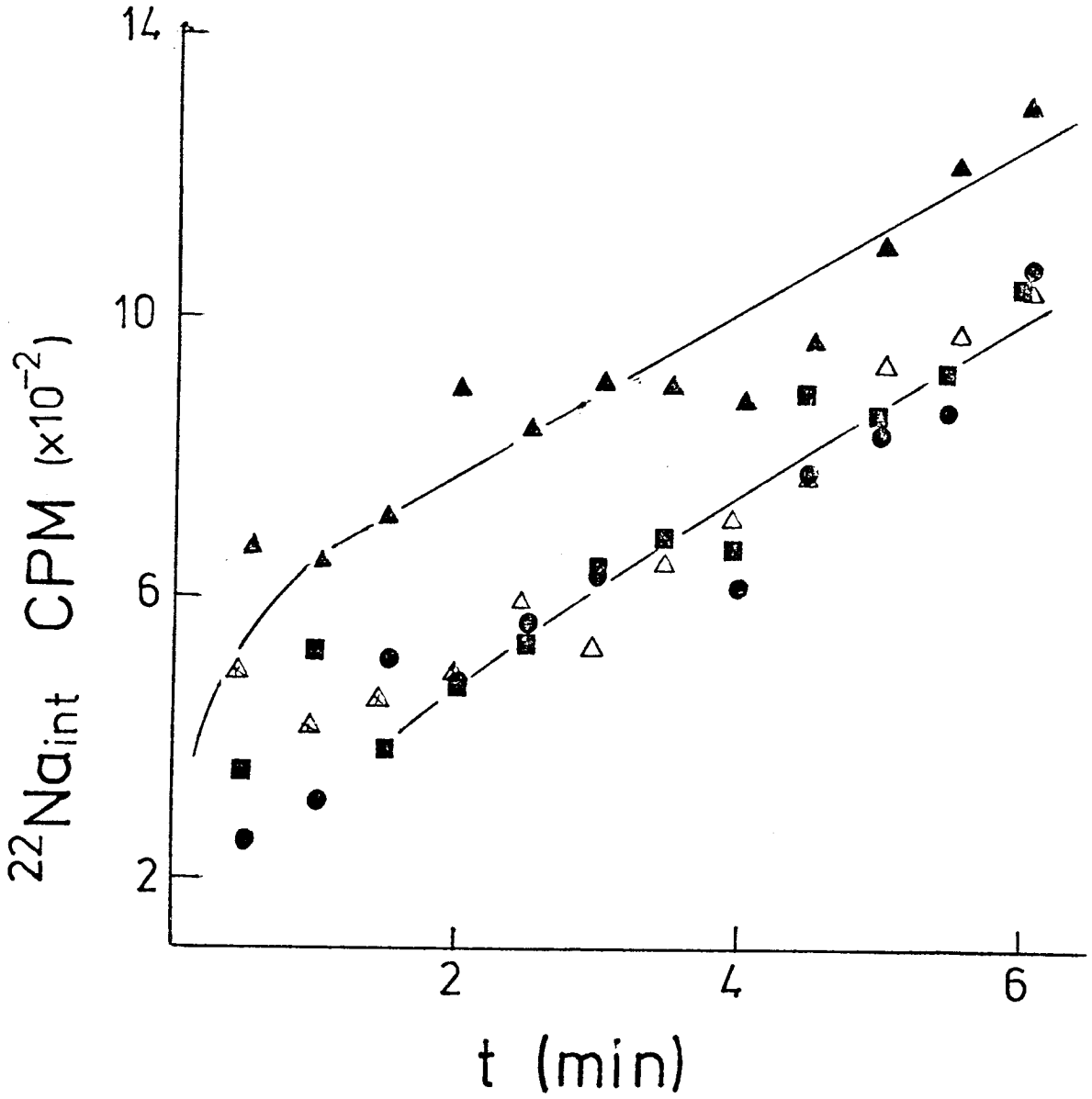
In an effort to further characterize the results of the *in vitro* system as agreeing with known *in vivo* responses, the phenomenon of desensitization was studied.Desensitization is a phenomenon whereby the post-synaptic membrane becomes refractory to agonists (25). Resensitization occurs slowly after complete removal of the drug. Both Carb and Ach induce desensitization. To test for desensitization in *T. californica* membrane fragments, an experiment was done on the uptake of $^{22}Na^{+}$ as a function of addition of Carb before or simultaneous to the addition of $^{22}Na^{+}$ to a nonradioactive preparation. Fig. 7 shows the results of such an experiment. Clearly Carb no longer enhanced the uptake of $^{22}Na^{+}$ when preincubated with the membrane fragments before addition of $^{22}Na^{+}$.

Another experiment was performed to test for both desensitization and resensitization in the membrane fragments. Five aliquots of a single nonradioactive membrane fragment preparation were treated as follows. One, the control, was diluted 20-fold into nonradioactive Ringer's, 2.5 hours were allowed to elapse, and then aliquots were taken every 10 seconds and measured for radioactivity remaining in the microsacs. The second was diluted 20 times into Ringer's containing 35 μM Carb. 146 minutes later 6 aliquots were taken 30 seconds apart. Then a small amount of Carb was added sufficient to make the final Carb concentration 70 μM, and aliquots were taken every 10 seconds thereafter. The third was treated identically using Ach instead of Carb. The fourth was diluted 20x into Ringer's, 2.5 hours elapsed and then a small amount of Carb was added to make the final Carb concentration 35 μM, and aliquots were taken every 10 seconds thereafter. The fifth was treated identically using Ach instead of Carb. The

FIGURE 7



Uptake of $^{22}Na^{+}$ by Torpedo microsacs. (●) $^{22}Na^{+}$ added at t=0. (▲) id. + 35 μM Carb. (■) id. + preincubation in 175 μM dTC prior to simultaneous addition of $^{22}Na^{+}$ and Carb. (Δ) preincubation with 35 μM Carb prior to addition of $^{22}Na^{+}$.

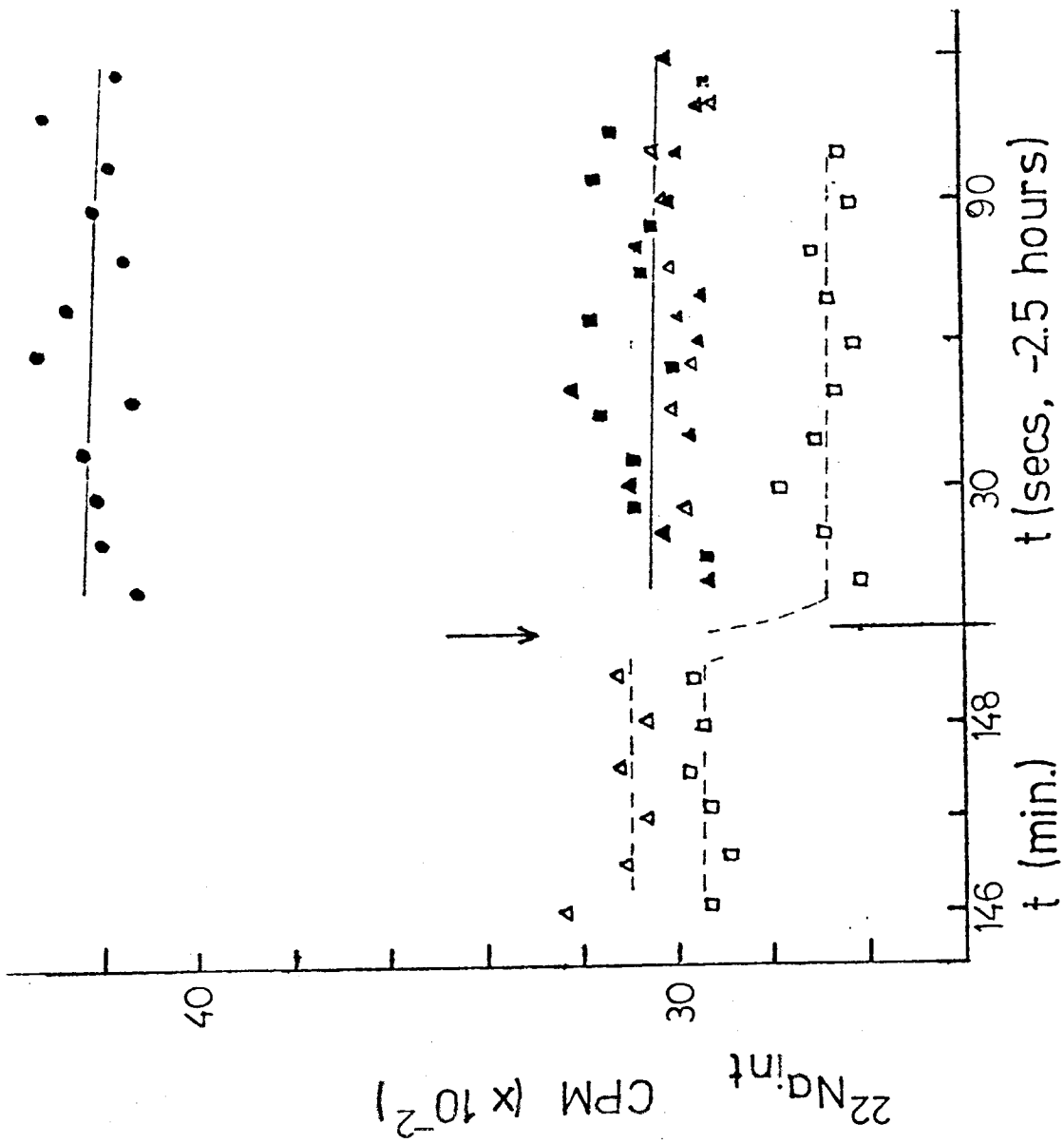

14
10
6
2
$^{22}Na_{int}$ CPM ($\times 10^{-2}$)
2
4
6
t (min)

results of that experiment are shown in Fig. 8.

In that experiment the amount of excitability (i.e. the decreased CPM levels relative to the control) produced by Ach and Carb at 35 μM is roughly the same. The amount of excitability produced is the same regardless of whether the preparation is initially diluted into agonist or is initially diluted into Ringer's and agonist added 2.5 hours later (to the same concentration). Apparently Ach excites the preparation before it is all hydrolyzed by the acetylcholinesterase (Achase) present (26). Addition of more Carb 2.5 hours later to the sample initially diluted into Carb does not result in further excitability. Addition of Ach 2.5 hours later to the sample initially diluted into Ach does result in further excitability. The implications of these results will be discussed in the Discussion section.

FIGURE 8

$^{22}Na^{+}$ efflux from T. californica microsacs. Times represented are those following dilution 20X at t=0. (●) dilution into Ringer's at t=0, no effector added at t=2.5 hours. (▲) dilution 20X into Ringer's at t=0, with addition of Carb at t=2.5 hours such that the Carb concentration is 35 μM. (■) id. using Ach instead of Carb. (Δ) dilution 20X at t=0 into Ringer's + 35 μM Carb. At arrow 35 μM Carb was again added. (□) id. except Ach was used instead of Carb.


$^{22}Na_{int}$ CPM (x 10^{-2})
40
30
146
148
30
90
t (min.)
t (secs, -2.5 hours)

DISCUSSION

AchR-rich membrane fragments derived from the electric organs of the marine electric ray Torpedo californica respond in vitro to cholinergic agonists by increasing their permeability to $^{22}Na^{+}$. Cholinergic antagonists block this effect. Histrionocotoxin, known to partially block in vivo permeability effects of agonists, also partially blocks the in vitro response.

The dose-response curve obtained in vitro from Carb agrees well with those obtained in vivo from eel electroplaques in regard to its shape and the apparent affinity for Carb of the AchR. Studies done on the isolated AchR and on membrane fragments from T. californica have indicated no positive cooperativity in the binding of Carb to the AchR exists (27). Therefore the cooperativity in the permeability change observed both in vivo and in vitro must occur sometime between the binding of agonist and the gross permeability change observed. Changeux suggests that perhaps tha AchR's are arranged in oligomeric clusters within the postsynaptic membrane in such a manner that cooperativity in ion channel opening results somehow from repeating lipoprotein units (28). While this may indeed be the case , to conclude on any one model for cooperativity is premature at this time. Whatever the explanation for cooperativity, this effect is retained in T. californica membrane preparations.

The results indicate that the in vitro response to agonists is rapid (less than 10 seconds) and then appears to terminate, i.e. the efflux rate of $^{22}Na^{+}$ then follows that of the control. In vivo the permeability increase due to agonists is known to be quite rapid (on the order of

milliseconds)(29) and desensitization occurs quickly thereafter (within a few seconds, depending on various parameters)(25). The results indicate that similar effects seem to be occurring *in vitro*.

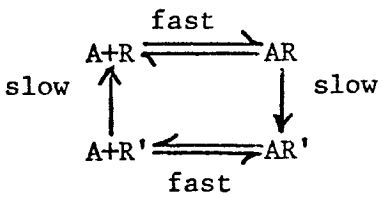
Fig. 7 shows that when Carb and $^{22}Na^{+}$ are added simultaneously to non-radioactive membrane fragments there occurs an initial rapid increased rate of $^{22}Na^{+}$ influx. However, when the microsacs are preincubated with the same amount of Carb prior to addition of $^{22}Na^{+}$ no increased rate of influx occurs. Thus it would appear that preincubation of the membranes with Carb desensitizes the AchR's.

As mentioned in the Results Fig. 8 shows another desensitization experiment. Several important characteristics of the flux system can be noted in these results. Regardless of whether agonist is present upon initial dilution of the membrane fragments or is added 2.5 hours after dilution the induced excitability is the same. Thus the increase in permeability is indeed rapidly occuring and then terminates, i.e. desensitization is manifested. When diluted initially into Ringer's containing 35 μM Carb the membrane fragments excite, desensitize and remain desensitized; addition of more Carb 2.5 hours later does not evoke more efflux. However, when initially diluted into 35 μM Ach, the microsacs apparently excite, desensitize, the Ach becomes hydrolyzed by the Achase present, the microsacs resensitize, and excitability is again elicted by a second addition of Ach. It seems that the microsacs desensitize before all of the available $^{22}Na^{+}$ can efflux. The fact that the excitability produced by a second addition of Ach is not as great as the initial one can probably best be explained by one of 3 ways. (1) The CPM levels of $^{22}Na^{+}$ seen after the second addition of Ach represent the equilibrium levels, i.e. where $[^{22}Na^{+}]_{int} = [^{22}Na^{+}]_{ext}$ and

thus there is no longer any driving force for the efflux of $^{22}Na^{+}$. (2) Upon initial dilution into Ach enough $^{22}Na^{+}$ has effluxed such that upon subsequent addition of Ach the driving force for $^{22}Na^{+}$, which is a function of $[^{22}Na^{+}]_{int}/[^{22}Na^{+}]_{ext}$, is lowered, and thus during the time the AchR is in an "open" state less $^{22}Na^{+}$ effluxes. (3) Choline, a product of hydrolysis of Ach by the Achase (17), may act as an antagonist and/or a desensitizer of the AchR.

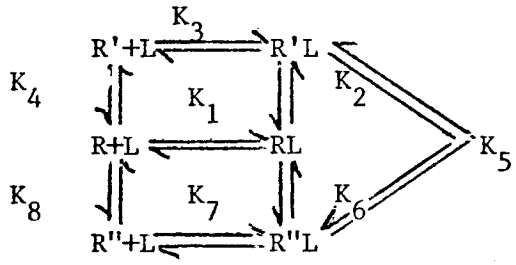
It has been shown that pretreatment of the AchR with cholinergic agonists results in an apparent affinity increase for the agonist (17,30). This conversion to a high-affinity form is reversible and dependent on severable parameters (ligand concentration, Ca^{2+} ions, temperature, etc.). Several workers have proposed that the low-affinity state corresponds to the sensitized AchR, while the high-affinity state corresponds to the desensitized AchR. The time course of this effect, as well as the dependence on ligand concentration, temperature, and Ca^{2+} are reminiscent of <u>in vivo</u> desensitization.

To explain these phenomena Katz and Thesleff (1957) proposed the following cyclic model for the action of the AchR:



where AR represents the agonist-receptor complex in the active (open) form and R' represents the desensitized receptor. Changeux and co-workers have done binding studies which support this model (30, 31), assuming

the high-affinity state corresponds to the desensitized one and vice-versa. However, recent work by Raftery and co-workers (10) seems to indicate that with T. californica AchR, the antagonist bis-(3-aminopyridinium)-1,10-decane diiodide can also convert the AchR to a form of high affinity for Carb. Therefore they propose the following minimal model for AchR action:



where R denotes the receptor in its resting state, RL an initial receptor-ligand complex, R' the open (depolarizing) state, and R" the desensitized (high-affinity) state of the AchR. This model differs from the Katz and Thesleff one in that the receptor need not necessarily go through an active conformation before assuming a desensitized one, since antagonists also induce conversion to the high-affinity form of the AchR. Experiments utilizing the in vitro excitability system should help to differentiate the two models. The effects upon inhibition of excitability of membrane fragments as a function of preincubation of membrane fragments with antagonists prior to addition of agonists versus simultaneous additions of both should help in defining a model for desensitization.

Whether the high-affinity state of the AchR is equivalent to the desensitized state, and the low-affinity to the sensitized, is not entirely clear at this time. However, binding experiments done in our lab have shown that membrane fragment preparations which did not show excitability were also predisposed to be in the high-affinity state for Carb. Also, in 3

out of 5 cases where the affinity state of a preparation that was shown to be excitable was measured, the AchR was in the low-affinity state prior to ligand addition. Thus it would appear that the high-affinity state of the AchR corresponds to a desensitized state, and a low-affinity state might correspond to a sensitized state.

There has been considerable interest recently on the effects of local anaesthetics on ligand binding to the AchR (32). Work has also been done on ligand-binding effects of chemical modification of various sites on the AchR (33). Such studies, together with measurement of these effectors' influence on increased ion flux from microsacs, should help elucidate the chemical events eliciting postsynaptic depolarization.

The in vitro system has several advantages over physiological systems: (1) The environment of the system can be well controlled. (2) The system is well defined. (3) The concentration of AchR can be quantitated using ^{125}I-α-Bgt. (4) The permeability properties of single ionic species can be investigated. (5) In cases where physiological measurements of the ionophore properties of the AchR are not feasible, such as in Torpedo, those properties need not be precluded from study.

REFERENCES

1. Hall, Z. W. Ann. Rev. Biochem. 41, 925 (1972).
2. Cohen, J. B. and Changeux, J.-P. Ann. Rev. Pharmacol. 15, 83 (1975).
3. Rang, H. P. Quart. Rev. Biophys. 7, 283 (1975).
4. Grundfest, H, in Sharks, Skates and Rays, Gilbert, P. W., Matthewson, R. F. and Rall, D. F., Ed., Baltimore, Johns Hopkins Univ. Press (1967).
5. Bennett, M. V. L. Ann. Rev. Physiol. 32, 471 (1970).
6. Bennett, M. V. L. in Fish Physiology, Vol. V, Hoar, W. S. and Randall, D. J., Ed., New York, N. Y., Academic Press (1971).
7. Vandlen, R. and Raftery, M. A., submitted to Biochem. (1976).
8. Claudio, T. and Raftery, M. A., submitted to PNAS (1976).
9. Raftery, M. A. et. al. Sixth International Congress of Pharmacology, Helsinki, Finland (1975).
10. Lee, T. et. al. submitted to PNAS (1976).
11. Raftery, M. A., et, al. in preparation (1976).
12. Michaelson, D. M., et. al. J. Supramol. Struct. 4, 419 (1976).
13. Kasai, M. and Changeux, J.-P. J. Membrane Biol. 6, 1 (1971).
14. Hess, G. P. et. al. PNAS 11, 4371 (1975).
15. Schmidt, J. and Raftery, M. A. Anal. Biochem. 12, 3593 (1973).
16. Michaelson, D. and Raftery, M. A. PNAS 71, 468 (1971).
17. References in Nachmanson, D. The Chemical and Molecular Basis of Nerve Activity, New York, Academic Press (1959).

18. Waser, P. G. in Curare and Curare-Like Agents, Bovet, D. D., Ed., Elsevier, Amsterdam (1959).

19. Waser, P. G. Hely. Physiol. Pharm. Acta 11, suppl. 8 (1953).

20. Chang, C. and Lee, C. Y. Arch. Int. Pharmacodyn. Ther. 144, 241 (1963).

21. Kato, G. and Changeux, J.-P. Mol. Pharmacol. 12, 92 (1976).

22. Albuquerque, E. X. et. al. J. Pharmacol. and Exper. Ther. 2, 513 (1974).

23. Del Castillo, I. and Katz, B., Proc. Roy. Soc. B 146, 339 (1957).

24. Brown, W. C. and Hill, A. V. Proc. Roy. Soc. (London) B 94, 297 (1922-1923).

25. Katz, B. and Thesleff, S., J. Physiol. 138, 63 (1975).

26. Vandlen, D. (unpublished observations).

27. Deutsch, J. in CIT Ph.D. Thesis.

28. Changeux, J. -P. et al., PNAS 57, 335 (1967).

29. Fatt, D. and Katz, B., J. Physiol. 115, 320 (1951).

30. Sugiyama, H. and Changeux, J. -P., Eur. J. Biochem 55, 505 (1975).

31. Weber, M. et al., PNAS USA 9, 3443 (1975).

32. Cohen, J. et al. Mol. Pharmacol. 10, 904 (1974).

33. Karlin, A., Fed. Proc. 8, Vol. 32, 8, 1847 (1973).