The Action of Serotonin on Calcium-45 Efflux from the Anterior Byssal Retractor Muscle of *Mytilus edulis*

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ABSTRACT ^{45}Ca efflux was studied in resting anterior byssal retractor muscle. The data are described by a three-compartment system. The most rapidly exchanging compartment, with an average time constant of 7 min, contains about 0.9 mM Ca/liter muscle, and probably represents extracellular space. A second compartment, with a time constant of 83 ± 5 min, contains 1.2 mM Ca/liter, and may represent a membrane calcium store. The presence of a third, or more, compartments, probably representing sarcoplasmic reticulum and contractile proteins, is indicated by the fact that the final time constant is 10 times the 83 min time constant of the second compartment. Serotonin (5HT), on initial application, increases ^{45}Ca efflux from this third compartment(s). This effect has a typical dose-response relationship with a maximum response appearing at 10^{-7} M 5HT. In addition, removal of 5HT causes a secondary increase in ^{45}Ca efflux which has a maximum at a 5HT concentration of 10^{-7} M and declines at both higher and lower doses.

INTRODUCTION

The anterior byssal retractor muscle (ABRM) of the mussel, *Mytilus edulis*, is a smooth muscle which exhibits two distinct types of contraction. The muscle is capable of phasic contractions or tetanus in response to low frequency stimulation. The muscle is also capable of sustained contraction in the absence of continued activation, catch, in response to applied acetylcholine, or DC stimulation. Serotonin, 5-hydroxytryptamine (5HT), specifically relaxes catch within seconds of its application (Nauss and Davies, 1966; Twarog, 1967 a). The muscle is innervated by nerves containing 5HT (Twarog, 1967 b); the stimulation of these nerves brings about the relaxation of catch. Schadler (1966) showed calcium activation in glycerinated preparations of the muscle. Twarog (1966) has speculated that catch is mediated by increased levels of intracellular free calcium, and that 5HT exerts its effects by specifically lowering the level of intracellular free calcium. In addition to its relaxing

effect, 5HT modifies phasic contraction and the electrical activity accompanying it. In the presence of 5HT, excitability is increased (Hidaka et al., 1967); the ability of the muscle membrane to fire spikes is increased. Moreover, effective membrane resistance is decreased in the presence of increasing concentrations of 5HT (Hidaka et al., 1967). The action potential is not affected by tetrodotoxin, suggesting that excitation is accompanied by a calcium spike (Twarog, 1967 c). Nauss and Davies (1966) studied calcium efflux from whole muscle preparations and demonstrated increased efflux on application of 5HT, but the large size of their preparation and probable contamination from shell and byssal organ make kinetic interpretation difficult. Hagiwara and Nagai (1970) presented evidence for increased efflux during contraction elicited by high K^+ concentration. In view of these observations, a close examination of calcium efflux both under control conditions and in the presence of 5HT was of interest.

METHODS

The ABRM has its origin on the anterior portion of the shell and inserts in the byssal organ. For these experiments, the whole muscle was carefully removed from the animal after sectioning of the nerves and cutting out a square of shell at the origin. The muscle was dissected into fine bundles, elliptical in cross-section with a maximum width of 100–155 μ and a minimum width of 70–95 μ. These bundles can be maintained in good physiologic condition for 6–8 hr, as indicated by their ability to respond to phasic (2–4/sec) stimulation. Such preparations eliminate the problem of large diffusion distances and consequently allow for more accurate time resolution. In addition, the problem of contamination from shell and byssal organ can be overcome by a specially designed apparatus which has been previously described (Curtis, 1970). In essence, the chamber used for these experiments collects effluent from only the central 0.7 cm of a bundle of muscle cells. Effluent from the rest of the bundle and from shell and byssal organ are suctioned away. After dissection, the muscle bundle was equilibrated in fresh artificial seawater for 30 min. It was then carefully measured at several points along its length and positioned in a special low volume trough where it was suspended in seawater containing $^{45}CaCl_2$ (approximately 0.5 mCi/ml) for 2–3 hr. At the end of this influx period, a sample of the influx fluid was taken for counting and the chamber was flushed with nonradioactive seawater. The muscle bundle was then positioned in the efflux chamber. Design of the influx chamber and efflux chambers is such that at no time during this transfer is the muscle bundle required to pass through a liquid-air interface, a possible cause of damage to the muscle cells. The preparation was then continuously perfused with nonradioactive artificial seawater at a rate of approximately 30 ml/min. Effluent from the central 0.7 cm portion was collected in planchets, dried, and counted to within 5 % accuracy in a Tracerlab low background counter (ICN, Chemical Radioisotopes Div., Irvine, Calif.). For most experiments, the collection interval was 10 min, although a few were performed using 5-min collection intervals. At the end of each experiment, the muscle was tested to determine whether it would respond to phasic stimulation. Any muscle

which did not respond was not included in the calculations. The central centimeter portion was then cut out, placed in a planchet with a small amount of seawater, dried, and counted with the other samples. The artificial seawater used in these experiments contained the following (in millimoles per liter): NaCl, 428; KCl, 10; MgCl$_2$, 52; CaCl$_2$, 10, and was buffered to pH 7.2 at 15°C with 50 mM tris(hydroxymethyl)-aminomethane (Tris)–HCl. ^{45}Ca solutions were made up by replacing a known molar concentration of CaCl$_2$ with the same concentration of ^{45}CaCl$_2$. All experiments were done at 15°C.

RESULTS

Resting Efflux. Fig. 1 shows a typical resting efflux curve plotted on a semilogarithmic scale. On the ordinate, counts per minute in the effluent per minute of collection interval are plotted. The abscissa represents time during the washout. The curve can be resolved into two straight lines. The later part of the curve (60–200 min) is fitted by the method of least squares to a straight line with a time constant of 74 min. We interpret these points to be the efflux from a single compartment which we will call the intermediate compartment. The mean time constant for this compartment was 83 min ($\pm$ 5 min, standard error [SE]; number [n] = 9 muscle bundles). In the early phase of the efflux, 0–30 min, the data points lie above the extrapolation of this line, suggesting that an additional compartment is releasing ^{45}Ca. By subtracting points on the line from the points in the efflux, one obtains, again by the least square method, a straight line with a time constant of 7 min which can then be ex-
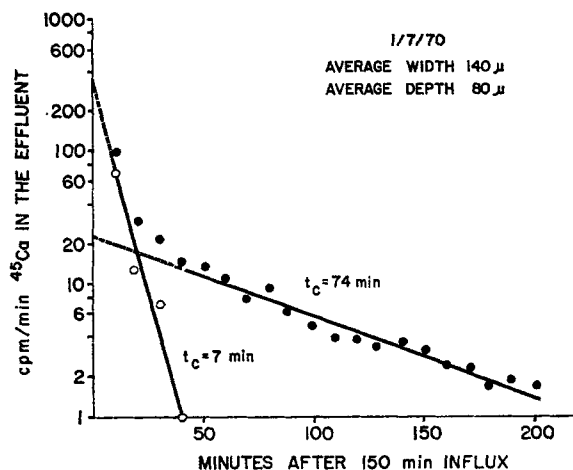

FIGURE 1. Efflux of ^{45}Ca into artificial seawater plotted as counts per minute in the effluent per minute collection interval vs. washout time. The open circles were obtained by extrapolating the 74 min time constant line back to zero (dashed line) and subtracting from early data points (closed circles). The lines were fitted by the method of least squares. The specific activity of the influx solution was 8.4 × 10^{-11} mM Ca/cpm. The final time constant is 833 min.

trapolated to zero time. The mean time constant for the fast compartment was 6.5 min (± 1 min, SE; $n = 9$ muscle bundles).

A time constant of 74 min, the intermediate component of Fig. 1, means that 1.35% of the ^{45}Ca in the compartment is leaving the compartment, and appearing in the effluent, each minute. At 200 min (the end of the experiment) the bundle was losing 2 cpm/min. Thus the compartment must have contained 2 cpm/min per 0.0135 min^{-1} or 148 cpm. When the bundle itself was cut out, dissolved, and counted, it contained 1660 cpm. Hence, there are more counts in the bundle than can be accounted for by the data points which describe the 74 min time constant. We have chosen to describe these additional counts per minute as a single time constant, although we realize that there may well be more than one compartment represented by the additional counts. So that the magnitude of this compartment can be compared between different bundles, it is described as a final time constant (the total counts per minute in the bundle divided by the rate of loss). In the case of Fig. 1, this time constant is 833 min. The mean for nine muscle bundles was 950 $\pm$ 122 min (SE).

Since the influx time (2–3 hr) is more than five times the average time constant for the fastest exchanging compartment, 6.5 $\pm$ 1 min, this compartment has reached isotopic equilibrium long before the beginning of the efflux phase. A simple direct method for determining the size of this compartment (Curtis, 1970) is to use the time differential of the standard equation for loss of radioactivity $P = P_0 e^{-kt}$, where $P =$ counts per minute at time zero, $k =$ rate constant, $t =$ time; $dP/dt = -kP_0 e^{-kt}$. Evaluated at zero time, this becomes $dP/dt_0 = -kP_0$. The plot seen in Fig. 1 is of dP/dt, i.e., the rate of loss of radioactivity from the bundle; therefore, the zero intercept of the line gives dP/dt_0, and the reciprocal of the time constant gives k. The average size of this compartment is 0.9 $\pm$ 0.20 mM/liter (SE) ($n = 4$). Potts (1958) obtained a value of 8.55 mM/liter as the total muscle calcium, so this fast compartment contains 10-12% of the total calcium. It may represent the extracellular space.

Analysis of the size of the second compartment is complicated by the fact that this compartment has not reached isotopic equilibrium. One method which partially overcomes this difficulty is to estimate the specific activity within this compartment, using the equation $P = P_0 (1 - e^{-t/\tau})$, where $P =$ counts per minute per mole Ca at any time t, $P_0 =$ counts per minute per mole Ca in the influx solution, and $\tau =$ time constant for efflux. (τ should equal the time constant for influx if there is no net gain or loss of Ca^{++}.) One inherent source of error in this method is the fact that the equation does not take backflux into account. Once one has obtained an estimate of the specific activity, P, of the compartment at the end of influx, it is possible to calculate the amount of Ca in the compartment from the expression $dP/dt_0 = -kP_0$. The average value for this compartment was found to be 1.23 $\pm$ 0.19 mM/

liter (SE) (n = 4). The second compartment thus contains approximately 15–20% of the total calcium. The remaining 65–70% of the total calcium is presumably contained in the third compartment or compartments with an average time constant of 950 min.

Effect of Serotonin (5HT). Fig. 2 shows the results of an experiment during which the preparation was perfused with 5HT (10^{-8} M), dissolved in the artificial seawater for limited periods of time. 5HT does not cause a relaxed muscle to contract. Approximately five times the ^{45}Ca collected in the previous
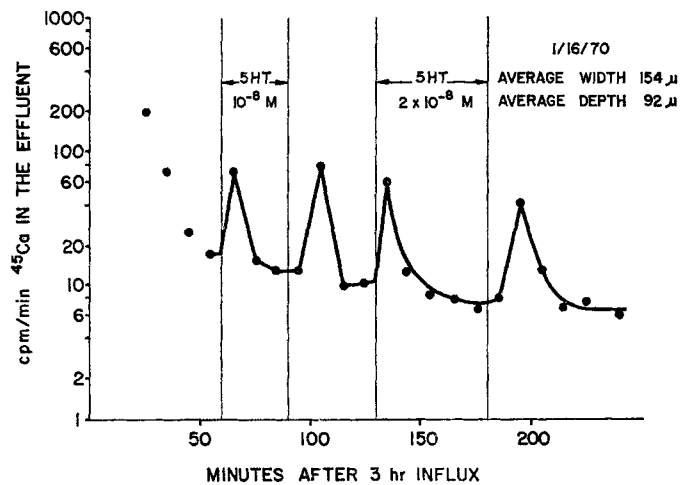

FIGURE 2. The effect of 5HT on ^{45}Ca efflux. The muscle is relaxed during the entire experiment. Note the increased efflux associated with both application and removal of 5HT. The line for the latter phase of efflux was not plotted as in Fig. 1 because the later points of the efflux curve were obtained under varying conditions (i.e., after application of 5HT) and no value for the first time constant could be obtained since there was no second time constant for extrapolation to zero. 5HT was applied twice: once at a concentration of 10^{-8} M from 60 to 90 min of the efflux and again at 2×10^{-8} M from 130 to 180 min of the efflux. The final time constant for this experiment was 200 min.

collection interval appears in the 10 min effluent collected immediately after the start of the 5HT perfusion. This marked increase in efflux soon subsides, even though the 5HT is still bathing the preparation. Upon removal of 5HT, however, there is a second increase in ^{45}Ca efflux. Now, five times the amount of ^{45}Ca collected in the last 10 min in the presence of 5HT is found in the effluent of the second 10 min collection period after removal of 5HT. This phase of increased efflux is also characterized by a very short duration. Calculation of the final time constant in this experiment gives a value of 200 min. The mean of the final time constant of muscles similarly treated with 5HT was 282 ± 86 min (SE). Comparison of this value with the average final time constant for control experiments (950 min) indicates that 5HT releases ^{45}Ca from

the most slowly exchanging compartment(s). That the second increase in efflux occurs only after removal of 5HT, and in fact is dependent upon removal of 5HT, is shown in Fig. 3. This figure represents an efflux curve in which the 5HT was allowed to perfuse the preparation for a considerably longer time (100 min). The ordinate of this curve is plotted as counts per minute remaining in the bundle rather than counts per minute appearing in the effluent to give a clearer picture of the magnitude of the loss of ^{45}Ca on application and removal of 5HT. No second increase in efflux occurs under these condi-
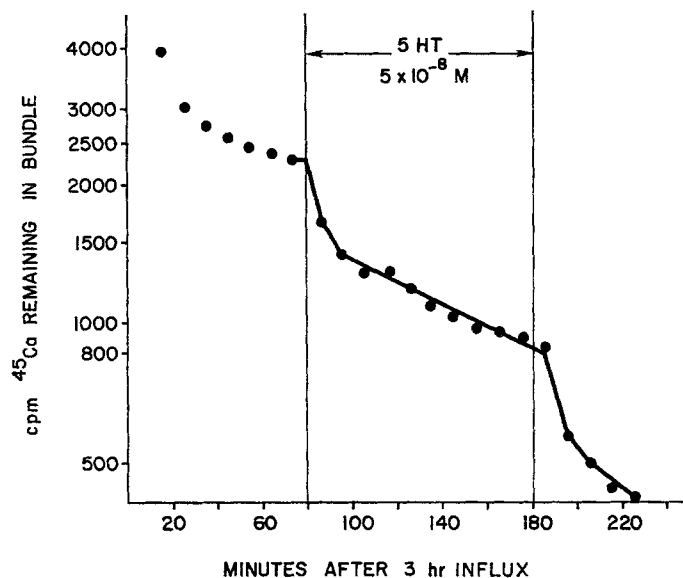

FIGURE 3.   The effect of prolonged application of 5HT on ^{45}Ca efflux. Curve is plotted as counts per minute remaining in bundle vs. time of washout. The counts per minute in the bundle was obtained by successive addition of counts per minute of ^{45}Ca lost during each collection interval to the counts per minute remaining at the time the experiment was dissolved and counted. A large efflux is now represented by a decrease in the total counts in the muscle. The second loss does not ocur until 15 min after removal of 5HT.

tions until the 5HT is washed from the preparation. Furthermore, this increase in efflux does not occur immediately after 5HT removal, but rather during the second collection interval after removal of the compound. Within the time resolution of the 10-min collection intervals, this delay is apparently independent of dose, i.e., the increased loss always occurs during the same time period regardless of the concentration of 5HT. Thus, 5HT has at least two distinct effects on the efflux of ^{45}Ca from these muscle bundles. It causes a sudden increase of ^{45}Ca efflux on application, and, 10–20 min after its removal, a second increase in efflux of ^{45}Ca. This kind of plot shows the marked depletion of ^{45}Ca brought about by 5HT treatment. If one extrapolates the data from 70 min

using the control time constant for the intermediate compartment (83 min) it can be calculated that about 1500 cpm would have remained in the bundle at the end of the experiment if 5HT had not been applied. With 5HT treatment less than $\frac{1}{3}$ of this value remains.

Using the compartment sizes obtained from control experiments and knowing the specific activity of the influx solution used in this experiment (1.9×10^{-10} mM/cpm) one can calculate the approximate counts per minute within each compartment at 75 min, the period just before the application of 5HT. In this experiment, the fastest exchanging compartment has been almost entirely depleted of its ^{45}Ca at the time of 5HT application, eliminating this compartment as a possible source of the increased efflux. At 75 min, the compartment which exchanges with a time constant of about 83 min contains only about 564 cpm. This means that the loss of almost 1000 cpm which occurs during the first 20 min after application of 5HT cannot come entirely from this intermediate compartment. Other experiments in which 5HT was applied much later during the efflux, so that this compartment contained even less ^{45}Ca, show little or no diminution of ^{45}Ca loss, confirming the impression that this compartment contributes little ^{45}Ca to the loss seen on application of 5HT. This leaves the most slowly exchanging compartment or compartments as the only possible source of the increased ^{45}Ca efflux. It can be estimated that this compartment in this particular experiment contained about 1600 cpm, at the time of 5HT application sufficient radioactivity to account for the initial increase in efflux.

The problem of ascertaining the immediate source of ^{45}Ca for the "delayed" increase in efflux is more complicated. Extrapolation of control data to calculate compartment contents is impossible after 5HT application since one cannot be certain that 5HT does not alter exchange rates for one or more of these compartments. Furthermore, increased ^{45}Ca exchange between compartments, for example, from the slowest compartment to the intermediate compartment, may be a possible action of 5HT. Such an exchange could markedly alter any theoretical calculation of compartment size. The calculated predictions of compartment size before 5HT application indicate that the original source of ^{45}Ca for the loss seen on 5HT removal must also be the most slowly exchanging compartment or compartments. This ^{45}Ca may move to other compartments before its release to the effluent, however. Dose-response curves for the two phases of ^{45}Ca loss are shown in Fig. 4. The ordinate represents per cent of the total ^{45}Ca remaining in the bundle which is lost during the first 10 min after 5HT application or the second 10 min period after 5HT removal (the time period during which the rate of efflux peaks). The abscissa represents molar concentration of 5HT. The graph shows that at doses less than 5×10^{-7} M the two responses run parallel. Above this dose, the per cent total ^{45}Ca lost after removal declines to only 9% at 10^{-3} M, while the initial
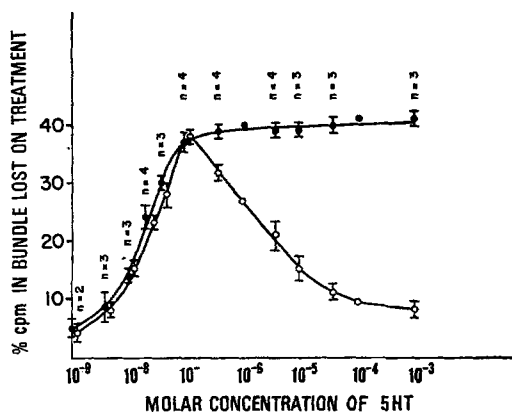
Figure 4. Dose-response curve for the per cent of the ^{45}Ca remaining in bundle which is lost on application of 5HT (closed circles) and per cent ^{45}Ca remaining in bundle lost on removal of 5HT (open circles).

response remains constant. The "initial" increase in efflux shows a more or less standard dose-response relationship, typical of many drug receptor reactions. The "delayed" increase in efflux on the other hand, reaches a peak, then declines as dose is increased.

DISCUSSION

The results from this study of ^{45}Ca efflux suggest that at least three compartments contain the exchangeable calcium in the ABRM, and that 5HT increases calcium efflux from at least one of these compartments, both on its application and on its removal. In ^{45}Ca efflux experiments on whole muscles, Hagiwara and Nagai (1970) found evidence for at least a two-compartment system in the ABRM of *Mytilus coruscus*. Our time constant for the fast component (6.5 ± 1 min) is in excellent agreement with that found in their experiments (6 min). Our middle compartment presumably corresponds to their slow compartment which has a slightly slower time constant, 108 ± 10 min, than does our middle compartment (83 ± 5 min). This discrepancy may be due to differences in species, but, more intriguingly, may be the result of the fact that their preparation was a whole muscle, and therefore undoubtedly contained many nerves while ours, due to the extensive dissection, was more thoroughly stripped of its nerve supply. Interestingly, there are physiological differences between nerve–whole muscle preparations and muscle bundles (Hidaka et al., 1967). The nerve-muscle preparation characteristically exhibits minimal catch, and applied 5HT has little or no effect on resting potential, junction potentials, spikes, or contraction. In muscle bundles, on the other hand, catch is more easily evoked by the appropriate stimuli, and 5HT prolongs junction potentials, lowers the threshold for spike discharge and

contraction, and decreases the effective membrane resistance. It may be that in whole muscle preparations, there is sufficient 5HT released from intrinsic nerves to prevent any additional electrophysiological effect of applied 5HT. This suggestion is somewhat supported by our data of ^{45}Ca efflux in the presence of 5×10^{-8} 5HT which shows a mean time constant for the intermediate component of 110 min, a value close to the control value (108 ± 10 min) of Hagiwara and Nagai. Alternatively, it may be that, in the presence of innervation, applied 5HT is more quickly inactivated so that its effects are diminished in the whole muscle preparation.

The anatomic positions of the postulated compartments are not totally clear. It would seem likely that the most rapidly exchanging compartment ($t_c = 6.5$ min) represents the extracellular space. An analysis by cutting and weighing of electron micrographs of the ABRM (Twarog, 1967 a, b, c) gives a value for the extracellular space of about 8%, which at 10 mM Ca in the artificial seawater would contribute 0.8 mM Ca/liter of muscle. The value calculated from the data is 0.9 mM/liter, which seems to be in good agreement. The intermediate compartment ($t_c = 83$ min), comprising 15% of the total muscle Ca, probably represents Ca bound to membrane structures. The evidence is mainly negative; every other excitable tissue studied has had a membrane Ca store. Efflux experiments (Curtis, 1970) on frog skeletal muscle assign 6% of the total Ca to the membrane compartment, but the ABRM has a much larger surface-to-volume ratio, so the larger percentage may be in keeping with data from other muscles.

The muscle contains one or more additional Ca compartments of which data is scanty because of the long average time constant (950 min). Two lines of evidence make it clear, however, that the increased ^{45}Ca efflux after application and withdrawal of 5HT comes predominantly from this compartment or compartments. First, analysis of the amount of ^{45}Ca remaining in each compartment just before 5HT application indicates that only the most slowly exchanging compartment(s) (time constant, 950 min) contains sufficient ^{45}Ca to account for the amounts lost. Second, in bundles treated with 5HT there is a marked reduction in the final time constant from an average of 950 min in untreated preparations to an average of 282 min in those treated with 5HT.

Our data show that 5HT causes large phasic changes in ^{45}Ca efflux, supporting to some extent the suggestion (Twarog, 1966) that 5HT abolishes catch and potentiates phasic activity by lowering levels of intracellular free calcium. On application of 5HT there is a prompt increase in the rate of calcium efflux. The peak of this increased efflux occurs within the first 5 min after 5HT reaches the muscle bundles, and the increase in efflux is completed within 20 min of application of the neurohumor. The magnitude of the increased loss of radioactivity follows a dose-response curve characteristic of first-order reaction kinetics and typical of many drug-receptor interactions.

Thus the time-response and dose-response relations strongly suggest that the increase in ^{45}Ca efflux seen on initial 5HT application is the result of an interaction of 5HT with receptors at or near the cell surface. The ^{45}Ca released by 5HT comes from the most slowly exchanging compartment(s), possibly reflecting an event at the reticular vesicles or the contractile proteins. In addition, the dose-response curve for the initial rapid efflux phase in resting muscle has its fastest rising phase very close to the doses of 5HT which optimally relax catch (Twarog, 1966).

The increase in efflux on 5HT removal, though difficult to interpret in terms of electrophysiological or mechanical data, is intriguing theoretically because it seems to represent a specific effector cell response to the removal of a neurotransmitter substance. This phase of increased ^{45}Ca efflux has a dose-response curve which peaks at 10^{-7} mM so that, beyond this dose, the percentage of the remaining radioactivity lost by the cells on 5HT removal becomes progressively less while the loss of radioactivity seen on initial application remains relatively constant.

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