described above. The material having the R_F value of 0.27 was identified as α-ecdysone, by means of HPLC and gas chromatography of trimethylsilyl derivative (*12*). Similar conjugates of 2-deoxy-α-ecdysone and of the compound having an R_F value of 0.35 were also found in extracts of ovaries in a comparable amount to the free forms.

Assuming that the molecular extinction coefficients of 2-deoxy-α-ecdysone and of α-ecdysone are the same as that of β-ecdysone, and neglecting the loss during purification, the content of the compounds in ovaries and in embryos was calculated from the peak areas in the chromatograms of HPLC by comparison with a standard curve constructed with authentic β-ecdysone (*7*). The content of free 2-deoxy-α-ecdysone in pharate adult ovaries is 0.31 μg/g, while that of the conjugated form is 0.46 μg/g. The content of free 2-deoxy-α-ecdysone in emerged adult ovaries is 0.22 μg/g, while that of the conjugated form is 0.26 μg/g. The content of 2-deoxy-α-ecdysone in diapausing embryos is 0.36 μg/g in a conjugated form.

The discovery of 2-deoxy-α-ecdysone in insect material is significant, since it lends support to the hypothesis that 2-deoxy-α-ecdysone is the immediate precursor of α-ecdysone (*9*).

Reevaluation of the role of conjugates of ecdysones seems necessary. Conjugates have been postulated to be inactivation products (*13*), but the presence of the conjugates of 2-deoxy-α-ecdysone and of α-ecdysone suggests that they are normal metabolic intermediates or storage forms, part of which may eventually be transformed into free α-ecdysone during the course of embryonic development (*7*).

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Transmitter Release During Repetitive Stimulation: Selective Changes Produced by Sr^{2+} and Ba^{2+}

Abstract. *The addition of Sr^{2+} or Ba^{2+} to the solution bathing the frog neuromuscular junction leads to an increased release of transmitter by each nerve impulse during and following repetitive stimulation. The mechanisms by which Sr^{2+} and Ba^{2+} increase release are not the same. Each ion appears to act selectively on a different process involved in transmitter release.*

The amount of transmitter released from a synapse by each nerve impulse varies as a function of previous synaptic activity (*1*). The factors which give rise to this variation in transmitter release have traditionally been distinguished from one another by differences in the duration of their effects. On this basis at least four processes (*2*) have been shown to increase transmitter release at the synapse of the vertebrate neuromuscular junction: a first component of facilitation which decays with a time constant of about 50 msec (*3*); a second component of facilitation which decays with a time constant of about 300 msec (*3*); augmentation which decays with a time constant of about 7 seconds (*4*); and potentiation (post-tetanic potentiation) which decays with a time constant of tens of seconds to minutes (*5*). Some of the factors involved in facilitation, augmentation, and potentiation of transmitter release appear to be different (*6*), but until now it has been difficult to distinguish among these processes except on the basis of their time constants of decay. We now report that Sr^{2+} and Ba^{2+}, two ions that can substitute for Ca^{2+} in evoked transmitter release (*7, 8*), have differential effects on the processes involved in transmitter release. While Sr^{2+} selectively increases the magnitude and time course of the second component of facilitation, Ba^{2+} selectively increases the magnitude of augmentation. These differential effects of Sr^{2+} and Ba^{2+} provide a new way to distinguish among the processes which increase transmitter release during repetitive stimulation and suggest that these processes can act relatively independently of one another.

End-plate potentials (EPP's) were recorded from the sartorius nerve-muscle preparation of the frog *Rana pipiens* by means of intracellular and surface recording techniques (*4*). Surface recording sums the responses from many endplates giving a good measure of average intracellular activity (*3, 9*). Standard frog Ringer (115 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 2.16 m*M* Na_2HPO_4, 0.85 m*M* NaH_2PO_4, 5 m*M* glucose, 0.03 m*M* choline) was modified by reducing Ca^{2+} to 0.35 to 0.6 m*M* and adding 5 m*M* Mg to decrease transmitter release and thus prevent muscle twitch. The effect of Ba^{2+} on transmitter release was examined by adding 0.05 to 1.0 m*M* Ba^{2+} to this control bathing solution (*10*). The effect of Sr^{2+} was examined by adding 0.2 to 0.6 m*M* Sr^{2+} to the control solution or by replacing the Ca^{2+} in the solution with 0.6 to 1.5 m*M* Sr^{2+}.

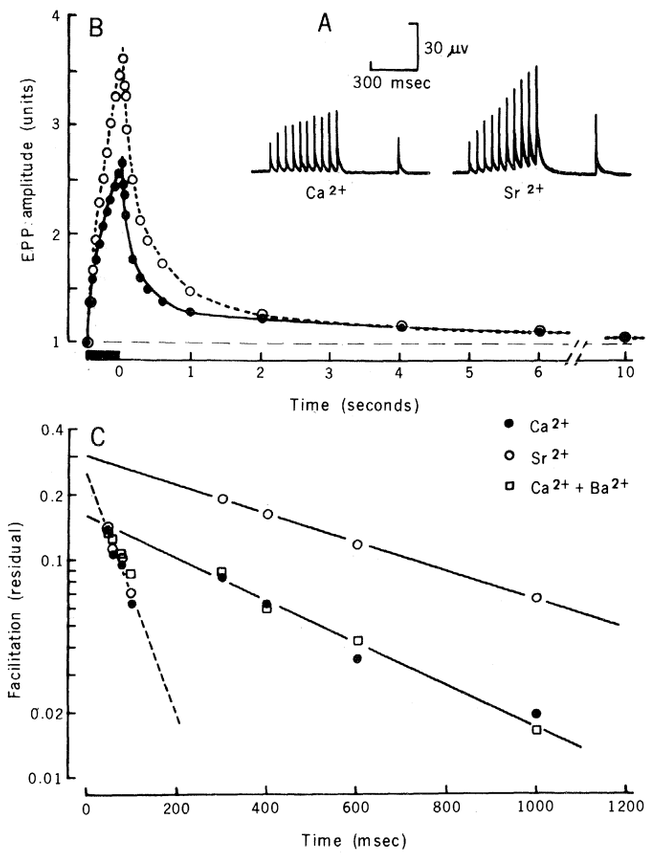
At the low levels of transmitter release in these experiments transmitter release is approximately proportional to EPP amplitudes (*4*). Facilitation, augmentation, and potentiation are all defined by the fractional increase in EPP amplitudes that they produce (*4, 11*). The contribution that each makes to increasing transmitter release was determined by successively peeling off the multiple exponentials used to describe the decay of the EPP amplitudes following the conditioning trains (*4*) or by using stimulation patterns which can test for each process during a conditioning train (*6*).

Figure 1 shows that replacement of the Ca^{2+} in the bathing solution with Sr^{2+} leads to an increase in the magnitude and time course of the second component of facilitation. The motor nerve was stimu-

lated with a series of conditioning and testing trials, each consisting of a conditioning train of 10 impulses followed by a single testing impulse. During the conditioning trains, EPP amplitudes increased to a greater magnitude in Sr^{2+} than in Ca^{2+} (Fig. 1, A and B), and after the conditioning trains the amplitudes of the testing EPP's decayed more slowly in Sr^{2+} than in Ca^{2+} (Fig. 1B). Estimates of the magnitudes and time constants of the two components of facilitation (*12*) following the conditioning trains in Sr^{2+} and Ca^{2+} are shown in Fig. 1C. Strontium almost doubled the magnitude of the second component of facilitation from 0.16 to 0.30 and increased its time constant of decay about 1.5 times from 440 to 650 msec (continuous lines). The Sr^{2+} had little effect on the first component of facilitation (Fig. 1C, dashed line) or on augmentation (Fig. 2C).

The Sr^{2+}-induced increase in the magnitude and time course of the second component of facilitation was always observed, came on rapidly (within the 5 to 10 minutes required to change solutions), was readily reversible, and was dose dependent over the tested range of 0.6 to 1.5 m*M* Sr^{2+}. The addition of small amounts of Sr^{2+} (0.2 to 0.6 m*M*) to a bathing solution containing 0.5 m*M* Ca^{2+} also gave rise to the Sr^{2+} effect. Using stimulation patterns which tested for facilitation during the conditioning train we found that the effect of Sr^{2+} on the second component of facilitation persists during 100 to 400 impulse conditioning trains. As was the case following the shorter conditioning trains, Sr^{2+} did not increase the magnitude of augmentation or potentiation following the longer conditioning trains (*13*).

Figure 2 shows that the addition of a small amount of Ba^{2+} to a bathing solution containing Ca^{2+} increases the magnitude of augmentation. The EPP amplitudes increased to a greater magnitude during the ten impulse conditioning trains recorded in Ca^{2+} + Ba^{2+} than during those recorded in Ca^{2+} alone (Fig. 2, A and B). After the conditioning train the EPP amplitudes decayed more slowly in Ca^{2+} + Ba^{2+} than in Ca^{2+} alone, but the form of the prolongation of decay was quite different from that induced by Sr^{2+}. Barium selectively increased the magnitude of augmentation following the conditioning train about 1.8 times from 0.26 to 0.47 as shown in Fig. 2C, but had little



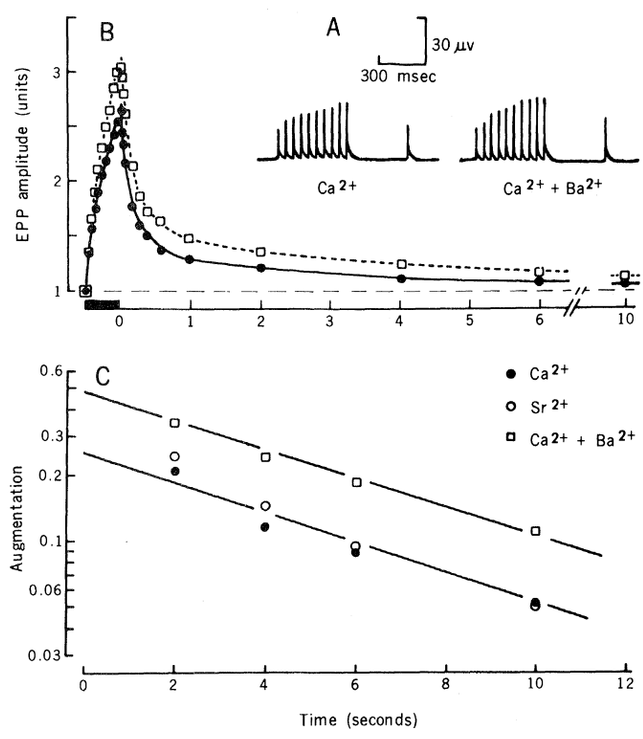


Fig. 1 (left). Strontium ions selectively increase the magnitude and time course of the second component of facilitation. The nerve was stimulated at a rate of 20 impulses per second for 10 impulses. Single testing impulses were applied from 50 msec to 10 seconds following each conditioning train, and a conditioning-testing train was applied once every 30 seconds. (A) Tracings of surface recorded EPP's obtained in bathing solutions containing 0.55 m*M* Ca^{2+} and then with 1.2 m*M* Sr^{2+} replacing the Ca^{2+} (*17*). The conditioning-testing interval was 400 msec. (B) Normalized amplitudes of surface recorded EPP's obtained during (horizontal bar) and following a series of conditioning trains are plotted against time. Solid line: predicted EPP amplitudes in Ca^{2+}. Dashed line: predicted EPP amplitudes in Sr^{2+} (*14*). The Sr^{2+} data represent the average response of over 5000 conditioning-testing trials from six preparations. Data were collected for three to six solution changes between Sr^{2+} and Ca^{2+} for each preparation. The control solutions contained 0.5 to 0.6 m*M* Ca^{2+} which was replaced with 1 to 1.5 m*M* Sr^{2+} for the Sr^{2+} data (*18*). The Ca^{2+} data from this series of experiments and from those described in Fig. 2 appeared the same and were averaged together so that the data in Figs. 1 and 2 could be compared. (C) Estimates of the residual substance responsible for the first (dashed line) and second (solid lines) components of facilitation following the conditioning trains in Figs. 1B and 2B are plotted semilogarithmically against time (*12*). Fig. 2 (right). Barium ions selectively increase the magnitude of augmentation. Same stimulation pattern as in Fig. 1. (A) Tracings of surface recorded EPP's obtained in bathing solutions containing 0.5 m*M* Ca^{2+} and then with 0.5 m*M* Ca^{2+} + 0.15 m*M* Ba^{2+}. (B) Normalized amplitudes of surface recorded EPP's. Dashed line: predicted EPP amplitudes in Ca^{2+} + Ba^{2+} (*14*). The Ca^{2+} + Ba^{2+} data represent the average response of over 2500 conditioning-testing trials from three preparations. Data were collected for three to four solution changes between Ca^{2+} + Ba^{2+} and Ca^{2+} for each preparation. The control solutions contained 0.45 to 0.55 m*M* Ca^{2+}; 0.15 to 0.25 m*M* Ba^{2+} was added to these solutions for the Ca^{2+} + Ba^{2+} solutions (*18*). (C) Estimates of augmentation following the conditioning trains in Figs. 1B and 2B are plotted semilogarithmically against time. The Sr^{2+} data point at 2 seconds is high because of the prolonged time course of facilitation in Sr^{2+}.

effect on the two components of facilitation (Fig. 1C).

Barium increased the magnitude of augmentation even more after longer conditioning trains of 100 to 400 impulses delivered at a rate of 20 per second. In 16 experiments of this type the average magnitude of augmentation increased from 1.2 ± 0.2 (mean ± standard error of the mean) in Ca^{2+} alone to 4.2 ± 0.9 in Ca^{2+} + Ba^{2+}, for an average increase of 3.5 times. Compared to the large increase in the magnitude of augmentation after the longer trains, Ba^{2+} had relatively little effect on the time constant of decay of augmentation or on the magnitude or the time constant of decay of potentiation. The Ba^{2+}-induced increase in the magnitude of augmentation was always observed, developed slowly (over about 30 minutes), was slowly reversible, and was dose dependent over the tested range of 0.05 to 0.5 m*M* Ba^{2+} in the presence of 0.5 to 0.6 m*M* Ca^{2+}.

The selective effects of both Sr^{2+} and Ba^{2+} could still be obtained in the presence of the other ion. The addition of Sr^{2+} to a solution containing Ba^{2+} increased the magnitude and time course of the second component of facilitation, while the addition of Ba^{2+} to a solution containing Sr^{2+} increased the magnitude of augmentation. As expected from these observations, the addition of both ions to the bathing solution led to a greater increase in transmitter release during repetitive stimulation than with either ion alone.

Changing the average level of transmitter release severalfold by adjusting the concentrations of Ca^{2+} and Mg^{2+} had little effect on estimates of facilitation, augmentation, or potentiation when compared to the dramatic and selective effects produced by Sr^{2+} and Ba^{2+}. These results suggest that the effects of Sr^{2+} and Ba^{2+} are specific and do not arise from general changes in the concentrations of divalent cations or from changes in the average level of transmitter release. The effects of Sr^{2+} and Ba^{2+} reported herein are of presynaptic origin resulting from changes in transmitter release, because the average amplitude of miniature EPP's remained constant during and following the conditioning trains, indicating that postsynaptic sensitivity remained constant.

The solid lines in Figs. 1B and 2B are the predicted increases in transmitter release during and following the conditioning trains, calculated by assuming that facilitation and augmentation accumulate and interact according to a model (*14*) that will describe transmitter release under a variety of conditions (*15*). Using this model with data obtained from the analysis of the decays of EPP amplitudes following the conditioning trains (Figs. 1C and 2C), we were able to describe accurately the increases in transmitter release during the conditioning trains. While other models are also compatible with these data, we believe it is significant that the effects of Sr^{2+} and Ba^{2+} on transmitter release during the conditioning trains can be predicted (dashed lines, Figs. 1B and 2B) by assuming that Sr^{2+} selectively increases the magnitude and time constant of the second component of facilitation, while Ba^{2+} selectively increases the magnitude of augmentation.

Thus, while both Sr^{2+} and Ba^{2+} increase transmitter release during repetitive stimulation, they appear to do so through quite different mechanisms. Since both Sr^{2+} and Ba^{2+} can enter nerve terminals and release transmitter (although not as effectively as Ca^{2+}) (*8, 10, 16*), it is possible that they act directly on the components in the nerve terminal involved in transmitter release. Alternatively, Sr^{2+} and Ba^{2+} may affect release indirectly by, for example, altering the rates of sequestering or the rates of entry of ions that affect transmitter release. Whatever the modes of action of Sr^{2+} and Ba^{2+}, the differential effects of these ions support the proposal that a number of presynaptic processes or factors mediate the effects of repetitive stimulation on transmitter release. Our results provide a new way to distinguish among these processes, and suggest that these processes can act relatively independently of one another.

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2. The term processes is not meant to imply mechanism but is used to refer to the characteristic dynamic properties of transmitter release that can be classified and separated mathematically by a kinetic analysis of changes in release during and following repetitive stimulation (*3–5*). The structural or chemical factors in the nerve terminal which give rise to the characteristic properties for each process are not yet known. A very fast decaying facilitation [B. Katz and R. Miledi, *J. Physiol. (London)* **195**, 481 (1968)], with a time course similar to that of evoked transmitter release, is not included in this analysis.
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10. It appears that Ba^{2+} has only a transient replacing action for Ca^{2+} in evoked transmitter release [B. Katz and R. Miledi, *Pubbl. Stn. Zool. Napoli* **37**, 303 (1969)]. Release was maintained in our experiments by leaving the Ca^{2+} in the Ba^{2+} containing solutions.
11. Augmentation is defined as $A(t) = [v(t)/v_0] - 1$, where $v(t)$ is the EPP amplitude at time t and v_0 is the control EPP amplitude. Facilitation and potentiation are defined in a similar manner. The definitions for each process apply only when the contributions of the other processes are insignificant or have been removed mathematically (*4*).
12. Estimates of facilitation depend on the assumed relationship between the various processes that increase transmitter release. The estimates in Fig. 1C were obtained by assuming that transmitter release $= [F_1(t) + F_2(t) + 1][A(t) + 1]$, where $F_1(t)$ and $F_2(t)$ are measures of the residual substances responsible for facilitation and $A(t)$ is the magnitude of augmentation. The magnitude of each process varies as a function of time. For example, the rate of change of augmentation with time is: $dA(t)/dt = aJ - kA(t)$, where a is the increment of augmentation added by each impulse J, and k is the rate constant for decay of augmentation where k = 1/time constant. The magnitude of potentiation was small after these short trains and was ignored. Previous studies have suggested a third power relation between facilitation and transmitter release [E. F. Barrett and C. F. Stevens, *J. Physiol. (London)* **227**, 691 (1972); M. R. Bennett, T. Florin, R. Hall, *ibid.* **247**, 429 (1975); S. G. Younkin, *ibid.* **237**, 1 (1974)], and a multiplicative relation between facilitation and augmentation [J. E. Zengel and K. L. Magleby, *Biophys. J.* **15**, 127a (1975)].
13. In some experiments the magnitudes of augmentation and potentiation following the longer conditioning trains in Sr^{2+} were decreased somewhat. These decreases may have arisen indirectly from a depletion of transmitter available for release due to the greatly increased transmitter release during the conditioning trains in Sr^{2+}.
14. Predicted EPP amplitudes were calculated from the equations in (*12*). Estimates of the magnitudes and time constants of decay of the increments of facilitation and of augmentation added by each nerve impulse were obtained from the magnitudes and time constants of each process after the conditioning trains by means of equations similar to those previously described (*3*). The values for these increments are: in Ca^{2+}, first component of facilitation, 0.12, 80 msec; second component of facilitation, 0.027, 440 msec; augmentation, 0.027, 6.0 seconds. In Sr^{2+} the values describing the second component of facilitation increased to 0.044 and 650 msec. In Ca^{2+} + Ba^{2+} the magnitude of the increment of augmentation increased to 0.049.
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17. The baseline is elevated slightly in Sr^{2+} because of an increased frequency of miniature EPP's that occurred in Sr^{2+} during and following the conditioning trains. The EPP amplitudes were measured as the distance from the baseline just before each EPP to the peak of the EPP to remove any effect of baseline shift on estimates of evoked transmitter release.
18. The concentrations of the divalent cations were adjusted to keep the control level of transmitter release (the quantal content of the first EPP's in the conditioning trains) about the same in the two different solutions. Similar effects were obtained when the divalent cations were adjusted to keep the average amount of transmitter released during the conditioning trains in the two different solutions about the same.
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