Kinetic Analysis of the Action of Chemical Modulators on Neuromuscular Transmission

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Abstract The kinetics of the direct action of 4-aminopyridine (4AP) and streptomycin (SM) on the mechanism of transmitter release were studied by recording the endplate potentials from curarized frog muscles using conventional microelectrode techniques, and by calculating the fractional release from the store of available acetylcholine quanta from the experiments with tetanic rundown during short train stimulations. To explain the tremendous facilitatory effect of 4AP on the fractional release and the antagonistic interaction of SM thereupon, it was postulated that 4AP and SM modify the transmitter output by combining with the Ca-dependent process X; i.e., 4AP and SM compete for the occupancy of the X site. A combination of 4AP or SM presumably modifies allosterically the action of the Ca-X complex, resulting in a profound augmentation of the evoked release of the available transmitter with the former and its depression with the latter. The theoretically derived equations from the above assumptions agreed reasonably well with the results obtained.

Aminopyridines have long been known to have a variety of actions on physiological functions. They have been shown to reduce the K conductance of the axonal membrane, exert an anticholinergic action on the neuromuscular junction (Sobek et al., 1968; Bowman et al., 1977; Harvey and Marshall, 1977), increase catecholamine release in a variety of tissues, stimulate respiratory activity (Fastier and McDowall, 1958), and produce strychnine-like convulsions (Fastier and McDowall 1958; Harvey and Marshall, 1977). The amplitude of the twitch tension of a muscle fiber is potentiated by these compounds (Fastier and McDowall, 1958; Bowman et al., 1977; Harvey and Marshall, 1977). In particular, 4-aminopyridine (4AP) and 3,4-diaminopyridine (34DAP) have a profound effect on the neuromuscular junction. By enhancing enormously the release of transmitter from the motor nerve terminal (Durant and Marshall, 1980), 4AP and 34DAP antagonize the d-tubocurarine (dTC) and antibiotic-induced neuromuscular blockades, and also relieve the long-lasting muscle paralysis produced by Botulinum.
toxin poisoning. Further, 4AP has been used clinically as a post-operative decurarizing agent (Stoyanov et al., 1976) and as a supplementary drug in the treatment of myasthenic syndrome (for literature on aminopyridines not cited here, refer to the review by Thesleff, 1980).

In the presence of 4AP, 34DAP, guanidine or tetraethylammonium (TEA), depolarization of the tetrodotoxin (TTX)-treated presynaptic nerve terminal causes a remarkable increase in inflow of Ca ions (Katz and Miledi, 1969a, b; Llinás et al., 1976a; Lundh and Thesleff, 1977; Illes and Thesleff, 1978; Lundh, 1978; Molgó et al., 1980). 4AP has no appreciable direct effect on either the time course of the endplate conductance change or the amplitude of miniature endplate potentials (Molgó et al., 1977; Lundh, 1978) but blocks selectively the K channels in the neuronal membrane (Ulbricht and Wagner, 1976; Yeh et al., 1976). Therefore, the facilitatory action of 4AP on the myoneural junction has been attributed to prolongation of the presynaptic spike as already shown by Benoit and Mambrini (1970) with TEA and UO2. This might increase the Ca influx into the motor nerve terminal, thereby facilitating the Ca-dependent process in depolarization-secretion coupling, and resulting in an increment of the release of acetylcholine (ACh). However, we are unable to definitely exclude the possibility of a direct facilitatory action of 4AP and 34DAP on the kinetics of either the voltage-sensitive Ca channel or a certain Ca-dependent release process in the motor nerve terminal as mentioned by several investigators (Lundh and Thesleff, 1977; Illes and Thesleff, 1978; Lundh, 1978; Molgó et al., 1979a).

The aminoglycoside antibiotics such as neomycin (NM) and streptomycin (SM) have been recognized to impair a variety of physiological functions including autonomic and neuromuscular transmissions, and auditory and renal functions (for reviews see Pittinger and Adamson, 1972; Sanders and Sanders, 1979). To explain the action of aminoglycoside antibiotics on neuromuscular transmission, it has been proposed that they interfere with the Ca-dependent process of depolarization-secretion coupling in the motor nerve terminal by competing with Ca ions for the specific receptor X on membrane of the nerve terminal to form a complex incapable of releasing the available ACh quanta (Vital Brazil and Prado-Franceschi, 1969; Prado et al., 1978).

The purpose of the present investigation was to elucidate the kinetics of the direct action of 4AP on the mechanism of transmitter release and the antagonistic interaction of SM thereupon by calculating the fractional release from the store of available ACh quanta (P) from the tetanic rundown of endplate potentials (EPPs) during short train stimulations. Some of the data has already been published in preliminary form (Maeno, 1980; Maeno and Enomoto, 1980).

**MATERIALS AND METHODS**

*EPP measurements and calculations of m and P.* The dTC-blocked sciatic

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nerve-sartorius muscle preparation of the Japanese frog, *Rana nigromaculata* was used. All experiments were conducted in an air-conditioned room (18–22°C). Intracellular recording of the EPPs from superficial muscle fibers was performed using conventional microelectrode techniques with glass capillary electrodes filled with 3 M KCl. The Ringer solution consisted of 115 mM NaCl, 2.5 mM KCl, 0.9 to 10 mM CaCl₂ as indicated, and 5 mM tris(hydroxymethyl)aminomethane. The pH of the solutions was adjusted to 7.2–7.3. Drugs used were 4-aminopyridine (Merck), streptomycin sulfate (Meiji), and *d*-tubocurarine chloride (Wako). The time course of recovery from depletion by stimulation was studied with the Ringer solutions containing 5 mM glucose and 0.1 mM choline chloride, but these were omitted in the other experiments.

Supramaximal short train stimulations (triple pulse stimuli) of 0.1–0.5 sec intervals were applied to the nerve fiber. Normally, the interval between the stimulation pulses was 0.1 sec throughout the year. However, the level of transmitter output in some of the winter frogs was so diminished that depression of the second EPP was frequently obscured by a long-lasting phase of potentiation which accompanied the first EPP. As a result, the calculated *P* often turned negative. Except for higher concentrations of 4AP, the interval between the stimulation pulses during the winter experiments was adjusted to between 0.1 and 0.5 sec to avoid obtaining negative values of *P*. Because of the tremendous increase in ACh release at higher concentrations of 4AP, a resting period of 1–2 min was allowed between train stimulations for recovery from depletion of the available store of ACh quanta. The concentration of dTC in each test solution was adjusted so that the first EPP response in a train was of almost the same amplitude. The 4AP concentration was increased stepwise, and the recordings of EPPs were made as a rule no more than three times in each solution. The amplitude of EPPs was corrected for non-linear summation (Martin, 1955).

When a train of indirect stimuli was applied to the curarized preparation, the amplitude of EPP diminished progressively due to a depletion of the store of the available ACh quanta. Since a fraction of the available store of the ACh quanta is released by a single nerve action potential, the quantal content of the first EPP (*m₀*) is given by:

\[ m₀ = P N₀, \]  

where *N₀* is a parameter probably related to the total number of ACh quanta available in the resting nerve terminal, and *P* is a parameter which has been termed as either fractional release or probability of evoked release. In the present experiments *P* was estimated from the apparent fractional release (*Pt*) calculated from the following equation:

\[ P_t = (V₀ - Vₜ)/V₀, \]  

where *V₀* and *Vₜ* are the amplitudes of the first and second EPPs in a train, respectively. As has been shown with experiments (Takeuchi, 1958; Otsuka et
al., 1962; Illés and Thesleff, 1978), \( P_t \) can be expressed as an exponential function of the stimulus interval \( (t) \); namely \( P_t = P_0 e^{-kt} \). When \( t \) is small (e.g., 0.1 sec) \( e^{-kt} \) becomes almost unity, and we obtain \( P_t = P \). Thus, we are able to estimate a fairly accurate value of \( P \) with this procedure.

As mentioned already by several authors (Illés and Thesleff, 1978; Lundh, 1978), the first nerve stimulus in a train of stimuli elicited a double EPP response when 20 μM or more of 4AP was added to 0.9 mM Ca Ringer. As a result, \( V_t \) was decreased due to the additional transmitter depletion by the double response following the first stimulus, leading to an overestimation of \( P \). Consequently, \( P \) under these conditions \( (P^*) \) was corrected with the equation \( P = 1 - (1 - P^*)V_t^*/V_{2t}^* \) where \( V_t^* \) is the amplitude of EPP response to the second stimulation recorded after the first double EPP response, and \( V_{2t}^* \) is the amplitude of EPP elicited by the third stimulation in a train.

When 0.5 mM 4AP was added to 0.9 mM Ca Ringer, every stimulus in a train evoked a double or triple EPP response. However, because of the marked depletion of the store of available ACh quanta, the reduction in the amplitudes of the second and third EPPs was so great that correction of the fractional release was no longer possible. In Ringer solutions containing 2.5 and 10 mM Ca no repetitive firing was observed at any concentration of 4AP tested.

During the present investigation, the effect of chemical modulators on the preparations in which the neuromuscular transmission was blocked on addition of 2 mM MgCl₂ to 0.9 mM Ca Ringer was analyzed using the Poisson equation. The nerve was stimulated at a rate of 2 Hz, and the mean quantal content of the EPP \((m)\) was calculated from the coefficient of variation of 100–200 EPPs. The EPP amplitudes were measured either by photographic means or by feeding the oscilloscope output to the digital memory (Iwatsu DM-305) coupled with the microcomputer (Hitachi H68/TR). There was a satisfactory correlation \( (r=0.9995) \) between the computer reading and photographed record of the same EPP.

**Working hypothesis for the accelerated evoked release with 4AP.** It is well established that the flow of Ca ions into the nerve terminal is essential for depolarization-secretion coupling. We assume the Ca ions combine with a specific structure \((X)\) in the nerve terminal and that the CaX complex formed would trigger a complicated chain of events of transmitter release. In the neuromuscular junction of the frog, this Ca-dependent process has been shown to involve the cooperative action of about four molecules of Ca (Dodge and Rahamimoff, 1967; Bennett et al., 1975; Miyamoto, 1975; Volle and Branisteau, 1976; Molgó et al., 1977, 1980). This might be interpreted to mean that the presumptive structure \( X \) is a single entity with four specific and independent sites, each capable of reacting with a single Ca ion. For release, all four specific sites must be occupied by Ca ions to form Ca₄X. In addition, it is assumed that the fractional release \((P)\) is related to the amount of Ca₄X; namely,
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\[ \frac{K_{Ca}}{P_{ca}} \]  

(3)  

\[ 4Ca + X \rightleftharpoons Ca_4X \rightarrow \text{evoked release}, \]

\[ P = P_{ca}[Ca_4X]/[X]_T, \]  

(3)

where \( P_{ca} \) is the parameter of evoked release for Ca, which is equal to the maximal \( P \) anticipated when all Xs are saturated with Ca ions, and \([X]_T\) is the total amount of X. From the above assumption, the value of \( P \) can be calculated from the following equation which is identical in principle to the familiar one described by Dodge and Rahamimoff (1967);  

\[ P = P_{ca}/(1 + K_{Ca}/[Ca])^4, \]  

(4)

where \( K_{Ca} \) is the dissociation constant for Ca.

Modification of Eq. (4) yields a linear double reciprocal relation between Ca concentration and \( P \); i.e.,

\[ \frac{1}{\sqrt{P}} = \frac{1 + K_{Ca}/[Ca]}{4\sqrt{P_{ca}}}. \]  

(4')

If the data are plotted on a double reciprocal scale, \( P_{ca} \) can be determined graphically from the intercept on the Y-axis, and \( K_{Ca} \) from the slope of this modified Lineweaver-Burk plot.

To explain the action of 4AP and the antagonistic interaction of SM thereupon, the authors would like to propose in the present paper the following simplified scheme.

(1) Chemicals which modify the transmitter output by acting on the above-mentioned Ca-dependent process X may be defined as chemical modulators. The chemical modulators may be divided further into two classes; i.e., accelerators and depressors.

(2) A group of drugs which have a facilitatory action fundamentally identical to that of 4AP are termed accelerators (As). An accelerator reacts with the Ca-bound structure X to form A \( \cdot \) CaX. The combination of A with CaX presumably modifies allosterically the action of the CaX complex to profoundly augment the evoked release. Thus, the fractional release in the presence of an accelerator may be considered to be the sum of two forms of release; i.e.,

\[ \frac{K_{Ca}}{P_{ca}} \]  

\[ 4Ca + X \rightleftharpoons Ca_4X \rightarrow \text{normal evoked release}, \]

\[ K_A \]  

\[ A + Ca_4X \rightleftharpoons A \cdot Ca_4X \rightarrow \text{accelerated evoked release}, \]

and so

\[ P = P_{ca}[Ca_4X]/[X]_T + P_A[A \cdot Ca_4X]/[X]_T. \]  

(5)

(3) Another group of chemicals called depressors (Ds) also react with the Ca-bound X sites, and thereby inhibit the ACh release; i.e.,
Therefore, the accelerator and depressor compete for occupancy of the Ca-bound X site. The fractional release ($P$) in the presence of both A and D is

$$P = \frac{(1 + K_{Ca}/[Ca])^4 + [A]/K_A + [D]/K_D}{P_A + P_A[A]/K_A},$$

where $K_A$ and $K_D$ are the dissociation constants for A and D, respectively. Families of the theoretical dose-release curves of 4AP in the absence or presence of SM were calculated from Eq. (6) and are presented in Figs. 5A and 7A, respectively. These curves fit satisfactorily the experimental data.

Modification of the above theoretical equation yields a linear relation between the concentration of depressor and the reciprocal of $P$; i.e.,

$$\frac{1}{P} = (1 + K_{Ca}/[Ca])^4 + [A]/K_A + [D]/K_D.$$

When $P = P_A$, we obtain $[D] = -K_D((1 + K_{Ca}/[Ca])^4 - P_{Ca}/P_A)$. Because $(1 + K_{Ca}/[Ca])^4 - P_{Ca}/P_A$ is almost unity under the present experimental conditions, this can be reduced to $[D] = -K_D$. Figure 8A shows such a relation. In accordance with the proposed theory, the straight lines all cross a single point, the co-ordinates of which are approximately equal to $[D] = -K_D$ and $1/P = 1/P_A$.

When $P_{Ca}$ is much smaller than $P_A[A]/K_A$ (in the case of 4AP, $[A] > 50 \mu M$), the above equation can be reduced to

$$\frac{1}{P} = \frac{1}{P_A} + \frac{K_A((1 + K_{Ca}/[Ca])^4 + [D]/K_D)}{P_A[A]}.$$

Thus, it is deduced from Eq. (7) that the effect of Ca and D on this approximately linear double reciprocal $P$ versus 4AP relation is to alter the slope without changing the intercept on the Y-axis where $1/P = 1/P_A$. As illustrated in Figs. 5B and 7B, the value of $P_A$ obtained from the Lineweaver-Burk plot was 0.92.

Equation (7) may be rearranged to yield the linear equation for the Eadie-Scatchard plot; i.e.,

$$[A] = \frac{P_A - P}{K_A((1 + K_{Ca}/[Ca])^4 + [D]/K_D)}.$$

Thus, except for the cases in which the contribution of the term $P_{Ca}$ cannot be ignored ($P/[A] > 70 \text{ mM}^{-1}$ in Fig. 8B), Eq. (7') predicts that the relation between $P$ and $P/[A]$ is linear, that the administration of D modifies the slope of this linear function, and that all the fitted straight lines intersect with the X-axis at $P = P_A$. Since $(1 + K_{Ca}/[Ca])^4$ is close to unity (about 1.2) under the present experimental conditions, the slope of this linear plot can be approximated to $-1/K_A$ when $[D] = 0$. Figure 8B shows such relations. Accordingly, we are able to estimate graphically the approximate values of $K_{Ca}$ and $P_{Ca}$ from the modified Lineweaver-Burk
plot (Eq. (4')), $K_A$ and $P_A$ with both the Lineweaver-Burk and Eadie-Scatchard plots (Eqs. (7) and (7'), respectively), and $K_D$ and $P_A$ by the Dixon plot (Eq. (6')).

RESULTS

Some properties of the accelerated evoked release with 4AP

4AP was found to increase enormously the amplitude of the EPP, in agreement with previous reports (Fig. 1). As a consequence, in higher concentrations of 4AP (4AP concentrations more than 0.1, 0.2 and 0.5 mM in 10, 2.5 and 0.9 mM Ca Ringer, respectively), the concentration of dTC had to be increased to 66 µM to avoid contraction of the muscle. This value is somewhat lower than 0.1–0.2 mM in the case of HEUSER et al. (1979). In the presence of 200 µM 4AP and 0.9 mM Ca, the amplitude of EPP after correcting for the effect of increased dTC concentration was found to increase to 33 times that of the control. This value corresponds to the degrees of chemical potentiation previously reported by HEUSER et al. (1979) and KATZ and MILEDI (1979).

It has been described previously with guanidine (OTSUKA and ENDO, 1960) and 4AP (ILLES and THESLEFF, 1978; LUNDH, 1978; HEUSER et al., 1979) that an enhancement of amplitude or quantal content of the EPP is associated with a marked increment in $P$. Similarly, in the present experiments with pulse train stimuli of 10 Hz, the remarkable increase in size of the EPP with 4AP (Fig. 1) was found to be accompanied by an extraordinary potentiation of the value of $P$ (Figs. 2 and 5A). Since $V=mv$ and $P=m/N$ ($m$, $v$ and $V$ are quantal content, quantal height

![Fig. 1. The effect of 4AP on the amplitude of EPP. Abscissa, the concentration of 4AP in µM; ordinate, the relative amplitude of EPP. The control EPP was taken unity. Each circle shows the average on two measurements. The external concentration of Ca was adjusted to 0.9 mM. Assuming that the amplitude of EPP is proportional to the value of $P$, relative amplitude of the EPP was calculated from Eq. (6) with the following parameters; $[Ca]=0.9$ mM, $[D]=0$ mM, $K_A=8.5$ µM, $K_{Ca}=0.50$ mM, $P_A=0.92$ and $P_{Ca}=0.14$. The theoretical curve fitted well with the data.](image-url)
Fig. 2. Effect of 4AP on the tetanic rundown of the EPP. The EPPs elicited with triple pulse stimulation of 100 msec interval were recorded on moving film. The concentrations of 4AP and dTC were from A to D, 0 and 4.4 (control), 10 and 22, 20 and 35, and 50 and 45 $\mu$M, respectively. The external Ca concentration was adjusted to 2.5 mM. Time scale, 5 msec; voltage calibration, 2 mV.

Fig. 3. Recovery from depletion of the available transmitter. Two EPPs generated with double pulse stimulation of various interval were superimposed on film. The interval of stimulations was from A to F, 0.1, 0.5, 1, 2, 3 and 5 sec, respectively. Perfusing solution contained 5 mM glucose, 2.5 mM Ca, 0.2 mM 4AP, 0.1 mM choline and 62 $\mu$M dTC. Time scale, 5 msec; voltage calibration, 2mV.

and amplitude of the EPP, respectively, and $N$ is the number of available ACh quanta), we obtain $V = vPN$. If $P_c$ and $V_c$ are defined to be respective values for
Fig. 4. The time course of recovery from depletion of the available ACh quanta. Abscissa, the interval of stimulation in sec; ordinate, the apparent fractional release \( (P) \) on logarithmic scale. The Ringer solution contained 5 mM glucose, 2.5 mM Ca, 0.2 mM 4AP, 0.1 mM choline and 62 μM dTC. Each circle represents an average of three measurements. The recovery process consists of two components shown by the broken lines.

the control, it may be assumed from the above relations that the relative amplitude of EPP \( (V/V_c) \) is equal to the relative value of \( P \), namely \( (P/P_c) \), so long as \( N \) and \( v \) remain constant. Based on this assumption, the theoretical curve for \( P/P_c \) was calculated using Eq. (6) and presented in Fig. 1. A good parallelism was found to exist between \( P/P_c \) and \( V/V_c \), as seen in the figure.

Since 4AP greatly enhances the stimulus-evoked transmitter release, the large number of transmitter-releasing sites thus emptied must be refilled with ACh vesicles newly recruited from the depot. This replenishment of the available ACh quanta from the depot can be estimated from the diminution of apparent fractional release \( (P) \) with elapse of time. As shown in Figs. 3 and 4, the depleted readily available ACh quanta recovered exponentially with a time constant of about 3.8 sec during the initial 5–6 sec, and more slowly thereafter. The time constant of the late phase of the recovery process found in the present experiments (5–11 sec; mean value of 6 cases, 7.8 sec) agreed with those reported previously on curarized preparations (Takeuchi, 1958; Otsuka et al., 1962; Illes and Thesleff, 1978).

In high 4AP Ringer, the resting period between trains was 1–2 min to allow recovery from depletion, and as a rule no more than three EPP recordings were made in each solution to avoid exhaustion of the transmitter. However, because of the marked release and slow replenishment of the available ACh quanta, the amplitude of the first EPP in a train gradually declined if the measurements of \( P \) were repeated many times in high 4AP Ringer. Despite the considerable reduction in the EPP amplitude under these conditions, the calculated \( Ps \) showed only an insignificant variation. The values of \( P \) were invariably smaller when the recordings of the EPPs were repeated with a shorter resting period.
Effect of Ca ions on the accelerated evoked release with 4AP

Since Ca ions are of primary importance to the ACh-releasing mechanism, the effect of Ca on the fractional release was investigated in some detail. In 0.9 mM Ca Ringer, the values of $P$ showed a greater variation, particularly when winter frogs were used. Such a variation of $P$ could be ascribed largely to a fluctuation of the amplitude of EPP, which is inevitable with low Ca concentrations. In the absence of 4AP, the mean value of $P$ in 0.9 mM Ca Ringer obtained from 23 muscle fibers was $0.027 \pm 0.035$ (mean ± standard error). In 2.5 mM Ca Ringer, the scatter in the values of $P$ was less marked. The mean value of 29 cases was $0.075 \pm 0.008$. Because of seasonal variation, the value of $P$ measured in 10 mM Ca Ringer was about 0.08 in winter and about 0.15 in summer. The average throughout the year was calculated to be $0.13 \pm 0.014$ (19 measurements). A further increment of Ca concentration to 15 mM increased $P$ only slightly. The parameters $K_{Ca}$ and $P_{Ca}$ could be determined graphically from the slope and the intercept on the Y-axis of the linear double reciprocal relation anticipated from Eq. (4'). $K_{Ca}$ and $P_{Ca}$ thus estimated from the present data were about 0.50 mM and 0.15, respectively. The value of $K_{Ca}$ is slightly smaller than the 1.1 mM reported by Dodge and Rahamimoff (1967) and Bennett et al. (1975).

In the present experiments, Ca ions were found to act synergistically with 4AP. As presented in Fig. 5A, an increase in the external Ca concentrations shifted the
dose-release relation of 4AP to the left. Similarly to the case with Ca ions, a linear relation was obtained with a double reciprocal plot of these data (Fig. 5B). The fact that the straight lines of Fig. 5B cross the Y-axis at the same point suggests that 4AP might modify the evoked release of the available ACh quanta by combining with a specific reactive structure for 4AP at the motor nerve terminal on which Ca ions act synergistically (see "working hypothesis" for details). With Eq. (7), the parameter of evoked release \( P_A \) for 4AP can be estimated from the intercept on the Y-axis of this double reciprocal plot. The value of \( P_A \) thus determined was 0.92. Suitable values of \( K_A \) and \( K_{Ca} \) calculated for the 4AP data of Fig. 5 were 7.5–8.5 μM and 0.50–0.55 mM, respectively.

**Antagonistic action of SM on the accelerated evoked release with 4AP**

Previous reports have shown that the neuromuscular block produced by various antibiotics was reversed by either Ca (FAIRHURST and MACRI, 1975; PRADO et al., 1978; SINGH et al., 1978) or aminopyridines (SINGH et al., 1978; MOLGO et al., 1979b). Thus, it was of interest to analyze how aminoglycoside antibiotics such as SM affect the neuromuscular transmission. With experiments on Mg-blocked preparations, it was found that the effect of lower doses of SM was a depression of the mean quantal content of EPP (m) as shown in Fig. 6A. At higher...
Fig. 7A (left). Effect of SM on the dose-release relation of 4AP. Abscissa, the concentration of 4AP in μM; ordinate, the fractional release. The concentration of SM was 0 (control), 0.34, 1.7, and 8.5 mM for the filled circles, triangles, squares and open circles, respectively. The Ca concentration was adjusted to 10 mM. The theoretical dose-release curves of 4AP were calculated from Eq. (6) with the following parameters; [Ca] = 10 mM, $K_A = 8.5 \mu M$, $K_{ca} = 0.50 \mu M$, $K_D = 0.65 \mu M$, $P_A = 0.92$ and $P_{ca} = 0.15$. Vertical bar indicates SE. The data are averages on 7–10 measurements.

Fig. 7B (right). Double reciprocal plot of the data presented in Fig. 7A. Abscissa, the reciprocal of the concentration of 4AP in 1/mM; ordinate, the reciprocal of the fractional release (1/P). The filled circles, triangles, squares and open circles represent the data obtained in the presence of 0 (control), 0.34, 1.7 and 8.5 mM SM, respectively. The theoretical straight lines were drawn from Eq. (7) with the following parameters; [Ca] = 10 mM, $K_A = 7.5 \mu M$, $K_{ca} = 0.50 \mu M$, $K_D = 0.60 \mu M$ and $P_A = 0.92$.

Concentrations of SM, the post-junctional sensitivity was depressed. These data suggested that the more sensitive target of SM might be the ACh-releasing process in the motor nerve terminal. On the other hand, SM was found, in the experiments with curarized muscles in 10 mM Ca Ringer, to be much less effective in depressing $P$ (Fig. 6B). ED$_{50}$ of SM was about 55 μM for $m$ in the Mg-treated preparation whereas it was as high as about 0.70 mM for $P$ in the curarized muscle. The latter figure is comparable to the values of ED$_{50}$ (0.74–1.8 mM) reported by several investigators (Fairhurst and Macri, 1975; Wright and Collier, 1977; Prado et al., 1978).

The effect of SM on the dose-release relation of 4AP is shown in Fig. 7A. SM antagonized the increase in evoked release caused by 4AP; i.e., SM shifted the 4AP-P curve to the right. However, the maximal value of the fractional release remained almost unaltered. Since the SM used in the present experiments was supplied in sulfate form, there is a possibility that the reduction of $P$ in the high SM solutions (8.5 mM SM Ringer) was somewhat overestimated by the presence of a large amount of sulfate. A replot of the data of Fig. 7A on the double reciprocal scale (Fig. 7B) shows clearly that the double reciprocal $P$ versus 4AP relation is approximately linear, and that the effect of SM is only to alter the slope of this linear
Lineweaver-Burk plot without changing the intercept on the Y-axis. These seem to support the hypothesis that the 4AP-SM interaction could be explained by the Michaelis-Menten kinetics as described in MATERIALS AND METHODS. When $K_A$, $K_{Ca}$, $P_A$ and $P_{Ca}$ were assumed to be 7.5–8.5 $\mu$M, 0.50 mM, 0.92 and 0.15, respectively, the value of $K_D$ derived from Fig. 7 was 0.60–0.65 mM.

Likewise, Eq. (6') predicts the presence of a linear relation between the concentration of SM and the reciprocal of $P$. The slope of this linear Dixon plot is also a function of 4AP concentration. As presented in Fig. 8A, this could be demonstrated with a similar replot of the data of Fig. 7A. The straight lines of this Dixon plot in Fig. 8A were calculated from Eq. (6') by adopting the following parameters; $K_A=7.3$ $\mu$M, $K_{Ca}=0.53$ mM, $K_D=0.50$ mM, $P_A=0.92$ and $P_{Ca}=0.15$. Except for the cases of low concentrations of 4AP ([A]<10 $\mu$M) the fitted straight lines agree reasonably well with the data. Similarly, Fig. 8B indicates that a linear relation can be obtained when $P/[A]$ is plotted against $P$. Except for the cases of large $P/[A]$ in the control, this is what is anticipated from Eq. (7'). The slope of this straight line is equal to $-1/K_A(1+K_{Ca}/[Ca])^4+[D]/K_D$ and the intercept on the X-axis gives $P_A$. The values of $K_A$, $K_{Ca}$ and $K_D$ thus estimated from Fig. 8B were 7.5 $\mu$M, 0.50 mM and 0.65 mM, respectively.

Fig. 8A (left). Dixon plot of the data given in Fig. 7A. Abscissa, the concentration of SM in mm; ordinate, the reciprocal of the fractional release (1/P). The concentration of 4AP was 10, 20, 50, 100 and 200 $\mu$M for the diamonds, inverted triangles, circles, triangles and squares, respectively. The straight lines were calculated from Eq. (6') with the following parameters; $[Ca]=10$ mm, $K_A=7.3$ $\mu$M, $K_{Ca}=0.53$ mM, $K_D=0.50$ mm, $P_A=0.92$, and $P_{Ca}=0.15$. Note that the straight lines all cross a single point where $[D]=-K_D$ and $1/P=1/P_A$.

Fig. 8B (right). Eadie-Scatchard plot of the data given in Fig. 7A. Abscissa, the fractional release; ordinate, $P/[A]$ in 1/mm. The filled circles, triangles, squares and open circles represent the data obtained in the presence of 0 (control), 0.34, 1.7 and 8.5 mm SM, respectively. The straight lines were drawn from Eq. (7') by adopting the following parameters; $[Ca]=10$ mm, $K_A=7.5$ $\mu$M, $K_{Ca}=0.50$ mM, $K_D=0.65$ mm and $P_A=0.92$. 

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Although the presynaptic effect of curare has been a subject of controversy, the results of some recent studies suggest that this drug has an effect on the process of transmitter release. Curare impairs short-term facilitation (Glavinović, 1979) as well as frequency facilitation (Maeno and Nobe, 1970), and accelerates the rate of tetanic rundown (Hubbard and Wilson, 1973). These results imply that curare may reduce or abolish the short-term facilitation which normally obscures the depletion of transmitter and that it also slows the rate of replenishment of the available store of ACh packets (Galindo, 1970). Such actions of curare are rather advantageous for the purposes of the present experiment, since they may make the value of P determined from the depletion of transmitter more precise.

The present data suggest the presence of a pharmacological interaction between chemical modulators. To elucidate how chemical modulators affect transmitter release, it is necessary to study the molecular characteristics of accelerators and depressors. The accelerators so far identified are 4AP (Lundh et al., 1977; Lundh and Thesleff, 1977; Lundh, 1978), 34DAP (Katz and Miledi, 1979; Molgó et al., 1980), and o-, m- and p-phenylenediamine (o-, m- and pPDA; Maeno, unpublished). Guanidine and TEA might be included in this category (Lundh et al., 1977; Lundh and Thesleff, 1977; Lundh, 1978; Katz and Miledi, 1979). Except for the cases of well-known guanidine and TEA, a common feature of the accelerators is characterized by a hexagonal skeleton with at least two nitrogen atoms attached at or within the positions of either 1 and 3 or 1 and 4 of the hexagonal ring (Fig. 9). The distance between two nitrogens is about 5 Å (4.4–5.1 Å). Recording of the nerve terminal action potential in guanidine and mPDA Ringer solutions suggests that prolongation of the electrical activity might not be the primary factor for the accelerated evoked release (Maeno and Enomoto, unpublished observation).

The molecular structures of the depressors are basically similar to those of the accelerators. As illustrated in Fig. 9, molecules of typical depressors, aminoglycoside antibiotics, consist of a hexagonal skeleton of deoxyinositol, nitrogen atoms

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Fig. 9. Comparison of the molecular structure of accelerator and depressor. Molecules are from left to right, 4AP, 34DAP, mPDA, SM and NM. Hexagonal skeleton of depressor consists of hydroxycyclohexane, and G and R denote guanido moiety and side chain, respectively. Note that chemical modulators shown in this figure all possess two nitrogen atoms attached at or within the position of either 1 and 3 or 1 and 4 of the hexagonal ring, distance of which is about 4.4–5.1 Å.

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attached at the 1 and 3 positions of the ring, and additional side chains linked at the
4, 4 and 5 or 4 and 6 positions. The distance between two nitrogens is also about
5 Å (4.9 Å). The side chains are very important for the activity of depressors, be-
cause detachment of glycosidically linked aminosugars from NM and SM reduces
the depressing potency both on neuromuscular transmission (Prado et al., 1978;
Enomoto, unpublished) and on $^{45}$Ca uptake (Lodhi et al., 1976). It might be of
interest to note in this connection that tobramycin, a recently introduced amino-
glycoside antibiotic, has been reported to augment the neuromuscular transmission
at higher concentrations (De Rosayro and Healy, 1978).

The results of binomial analysis of the Mg-blocked neuromuscular prepara-
tion indicate that 4AP, guanidine and TEA potentiate transmitter release from the
motor nerve terminal by increasing the average number of readily available quanta
for release ($n$) (Volle and Branisteau, 1976; Lundh, 1979; Molgó et al., 1979a;
Enomoto, unpublished). The probability of evoked release of the constituent
quanta ($p$) is not appreciably affected. Statistical analysis indicates that the de-
pression of the neuromuscular transmission produced by aminoglycoside antibiotics
can be ascribed primarily to a decrease in $m$, similarly to the results of Molgó
et al. (1979b). The results of binomial analysis by Enomoto (unpublished results)
suggest that such a diminution in $m$ is associated with a reduction in $n$; $p$ remained
substantially unchanged.

Since $m=pn$ and $P=m/N$, it is seen that $P=pn/N$. Consequently, it might be
explained under the present circumstances that a change in $P$ is correlated with an
alteration in $n$. The total storage number of the available ACh packets ($N$) would
presumably represent the total population of vesicles deployed along the active zone
in nerve terminals (Heuser et al., 1979), and the chemical modulators would alter
either directly or indirectly the fraction of that population in active form or state for
release ($n$). However, it is possible that $N$ might also be affected.

Though the present results are in good agreement with the proposed model,
they give no clue as to the site where the chemical modulators act. It is commonly
suggested in the literature that both the accelerators and depressors have some
relation with Ca ions. Accordingly, it might be assumed that these modify the vol-
tage-sensitive Ca channel (IlNAS et al., 1976b). Alternatively, the site of action
might be the active zone or the ACh-releasing site in the nerve terminal. Further,
there is the additional possibility that the chemical modulators exert their actions
by an effect on the binding of Ca ions with the intracellular Ca store such as mito-
chondria (AlNæs and Rahamimoff, 1975). In fact, radioactive tracer experiments
show that NM and SM suppress the uptake or binding of $^{45}$Ca with various subcel-
ular structures of the nerve and muscle (for further literature see review of Sanders
and Sanders, 1979).

Although the model proposed in this paper agrees satisfactorily with the pres-
ent results of 4AP-Ca and 4AP-SM interactions, it does not explain the previous
reports on the competitive interaction found between SM and Ca; i.e., NM and

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SM are less effective in higher Ca Ringer solutions (Fairhurst and Macri, 1975; Wright and Collier, 1977; Prado et al., 1978). When Eq. (6) is applied to the SM data of Prado et al. (1978), the calculated value of \( K_D \) varies with the Ca concentration (\( K_D \) is 0.3, 0.5 and 0.7 mM in 1.6, 1.8 and 2.0 mM Ca Ringer, respectively). Since transmitter release is a result of a complicated chain of events triggered by the nerve action potential, it is possible that the presumptive Ca-binding structure \( X \) in the present model is composed of two (or more) Ca-dependent processes, and that the chemical modulator reacts with one of them. In fact, the results of Prado et al. (1978) could be explained better if \( X \) is made up of two subsystems; one is involved in the first order and the other the third order reaction with Ca ions (Bennett et al., 1975). At present we have no definite evidence as to the nature and operation of the separate Ca-dependent subsystems. Therefore, \( X \) is treated as a single entity in this paper. The present model may be defective in this respect and may be the subject of revision in the near future.

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