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ON THE CALCIUM RECEPTOR THAT MEDIATES DEPOLARIZATION-SECRETION COUPLING AT CHOLINERGIC MOTOR NERVE TERMINALS

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1 The behaviour of the divalent cations Ca and Sr as agonists for receptors that mediate the synchronous evoked secretion of acetylcholine (ACh) was studied in the hope of determining whether the relationship between Ca binding and ACh secretion is determined only by the law of mass action or by the mathematical framework of receptor theory. Experiments were designed to evaluate the assumption that maximum effect requires occupation of all receptors by testing for the presence of spare Ca receptors on presynaptic terminals. Frog cutaneous nerve-muscle preparations were employed in conjunction with conventional electrophysiological methods.

2 Curves of log [Ca] or log [Sr] against the mean number of ACh quanta released ($\overline{m}$) were constructed to saturation. The log [Sr]-$\overline{m}$ relationship was shifted to the right and had a smaller maximum than the log [Ca]-$\overline{m}$ curve. This suggests that Ca has a higher efficacy than Sr and raises the possibility that spare binding sites are present for Ca.

3 As a qualitative test for spare Ca receptors, La^{3+}($\geq$0.5 μM) or 2-chloroadenosine (25 μM) was employed as an irreversible antagonist of the effects of extracellular Ca on evoked ACh release. Despite the irreversible blockade of a proportion of receptors, increases in the [Ca] overcame this antagonism and produced a parallel shift in the log [Ca]-$\overline{m}$ relation to the right. This suggests an apparent receptor reserve for Ca. Antagonism of Sr-mediated ACh release by either La^{3+} or 2-chloroadenosine could not be overcome by increasing the [Sr].

4 As a quantitative test for spare Ca binding sites, the equilibrium affinity constant for Sr(K_{Sr}) as a competitive inhibitor of Ca was determined and compared with values for K_{Sr} calculated by two other methods which invoke the spare receptor assumption. All three methods produced comparable results. (K_{Sr} = 0.24–0.27 mM^{-1}).

5 The equilibrium affinity constant for Ca (K_{Ca}) was calculated by comparing reciprocal plots of the concentrations of Ca that produce equal levels of ACh release in the presence and absence of La^{3+}(0.5 μM–3 μM). K_{Ca} was estimated to be between 0.02 and 0.06 mM^{-1}.

6 Efficacy (e), which is thought to reflect the ability of Ca or Sr once bound to receptors to support ACh release, was determined by the modified occupation theory of Stephenson (1956). The e_{Ca} was estimated to be 9–20 and e_{Sr} was 0.2–0.5.

7 The experimentally determined values for K_{Ca}, K_{Sr}, e_{Ca}, e_{Sr} along with the assumptions that spare Ca binding sites exist and that the non-linearities in the log [Ca] or log [Sr]-$\overline{m}$ curves are introduced *beyond* the sites of binding and efficacy were used to generate theoretical log [Me]-$\overline{m}$ curves. The theoretical relationships were similar to the experimental results.

8 The results suggest that spare Ca receptors are present at motor nerve endings and that receptor theory provides an accurate quantitative description of the lumped events between Ca binding and ACh secretion. The possible physical correlates of affinity and efficacy are discussed.

Introduction

The classical, quantitative studies on the action of alkaline earth cations in coupling nerve terminal depolarization to the synchronous, impulsive secretion of acetylcholine (ACh) quanta (Dodge & Rahamimoff, 1967; Meiri & Rahamimoff, 1971) have generally interpreted the relationship between cation binding and ACh secretion in accordance with the principle of mass action. This interpretation, which implies that ACh secretion is related in a predictable fashion to the extracellular concentration and affinity

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of the divalent metal ion (Me), is outlined as follows:

$$Me + X \overset{K_{Me}}{\rightleftharpoons} MeX \rightarrow e_{Me} \overset{\text{depolarization}}{\rightarrow\rightarrow\rightarrow\rightarrow} \text{ACh secretion}$$

In this scheme, the substrate Me (i.e. Ca or Sr) binds reversibly to a postulated site X at the nerve terminal membrane and occupies a certain fraction (y) of the total number of sites. Application of the law of mass action for the equilibrium condition gives

$$y_{Me} = \frac{K_{Me}[Me]}{1 + K_{Me}[Me]} \quad (1)$$

where K_{Me} is the affinity constant of the divalent metal ion with the hypothetical site X and y_{Me} is the occupancy for the particular Me species. Eqn. (1), describes a rectangular hyperbola and has been employed frequently to describe experimental results in physiology (Hill, 1909), enzyme kinetics (see Segel, 1975) and gas adsorption (Langmuir, 1918). In the above scheme, subsequent to occupation, depolarization of the membrane provokes the MeX complex, through a series of as yet little understood physicochemical processes, to produce the release of ACh. By the simple law of mass action, the magnitude of such secretion, as a fraction of the maximum level of secretion, is assumed to be proportioned to y in accordance with eqn. (1).

Using this approach in conjunction with conventional extrapolation methods (Lineweaver & Burk, 1934; Segel, 1975), Meiri & Rahamimoff (1971) found that Ca and Sr have approximately the same affinity for X ($\sim 1\ mM^{-1}$), but Ca, once bound to X, has higher efficacy (e) than Sr in unifying the individual quantal parcels into a multiquantal secretory event that is detected electrophysiologically as the endplate potential (e.p.p., Fatt & Katz, 1951). As the experimental results at low levels of ACh release could be fitted to a rectangular hyperbola using an apparent Hill coefficient (Brown & Hill, 1923) of four (i.e., by raising y to the fourth power) it was suggested that four Ca may cooperate in some manner to produce the release of a quantum of ACh (Dodge & Rahamimoff, 1967). Such cooperativity might endow the release mechanism with the properties of a regulatory enzyme, but as occupancy is a fractional value, a fourth power relation between y and release would greatly reduce the secretory response produced by a given occupancy as compared to a first power relationship. This power relation thus implies that a rather large fraction of sites need be occupied by Ca to produce a given level of ACh release.

In contrast to this interpretation, it is common for full agonists in complex biological systems to produce a maximal effect with quite small occupancies (Ariëns, 1979) and in turn leave the majority of receptors unoccupied or spare (Stephenson, 1956). In this regard it is possible that Ca, as full agonist, could have a low occupancy at receptor site X yet produce maximal levels of ACh release by virtue of an intricate chain of pre-secretory steps serving to amplify forcefully the initial binding signal. Implicit in this sort of relationship between occupancy and release is the presence of spare Ca receptors, a result which would cast disfavour on the simple mass action assumption for Ca-mediated ACh secretion. The purpose of the present study was to test for the existence of spare Ca receptors; and to examine whether the determined values for affinity and efficacy of the agonists Ca and Sr were in agreement with the spare receptor hypothesis. A brief account of some of these results has been presented to the Pharmacological Society (Silinsky, 1980a).

Methods

General

Isolated cutaneous pectoris nerve muscle preparations of frog (*Rana pipiens*) were dissected and superfused with flowing Ringer solution (for compositions see below). Preparations were transilluminated by a fibre optics system similar to that described by Dreyer & Peper (1974). Such illumination, in conjunction with a Wild M5 stereomicroscope used at 100 × magnification enabled nerve terminals to be located visually (cf. Dreyer & Peper, 1974). Stimulation pulses (Grass S88 and SIU 15) were delivered to the nerve through a suction electrode. Electrical potential changes were recorded intracellularly at endplate regions with glass microelectrodes filled with 3 M KCl, the reference electrode being a silver-silver chloride pellet (W.P. Instruments). Electrodes were filled by the fibreglass method of Tasaki, Tsukuhara, Ito, Wayner & Yu (1968) and had resistances ranging from 10 to 20 MΩ. Signals from the microelectrode were fed into a conventional preamplifier (W.P. Instruments-M701). The output of the preamplifier was delivered in parallel into an oscilloscope (Tektronix 5103 N), a pen recorder (Brush-Gould model 2200), and a computer of average transients (Fabritek). The output of the computer was displayed on another oscilloscope (Tektronix 564). In experiments where nerve terminal action potentials (n.t.ps) were studied, miniature endplate potentials (m.e.p.ps) were first localized extracellularly using a microelectrode filled with 1 M NaCl (1–3 MΩ resistances). The muscle was then curarized, the nerve stimulated and n.t.ps averaged by computer (e.g. Figure 2). M.e.p.p. amplitudes were determined from oscilloscope traces and m.e.p.p. frequency and muscle resting potentials were measured from pen records.

Composition of solutions

The standard Ringer solution contained (mM): NaCl 115, KCl 2, $MeCl_2$, X, with pH adjusted to 7.1–7.2 using $NaHCO_3$ 2, or N-2-hydroxyethylpiperazine-*N*-2 ethanesulphonic acid (HEPES)2. Normal Ringer contained 1.8 mM $CaCl_2$. In solutions of varying Ca and Sr concentration, osmotic compensation was not employed as the largest difference in the tonicity of the Ringer in these experiments, 31 mosmol/litre, does not affect $\bar{m}$ in frog muscle (Furshpan, 1956; Kita & Van der Kloot, 1977). In several experiments, the [Ca] was measured directly using a Ca titrator. In the majority of experiments in which $LaCl_3 \geq 20$ μM was used, the pH was reduced to ~6 by eliminating $NaHCO_3$ from the Ringer solution. Although this was necessary to prevent precipitation of La^{3+}, it also proved useful as it allowed subthreshold e.p.ps to be observed in a wider range of Ca and Sr concentrations than at normal pH. This effect may be due to the action of H^+ as a modest competitive inhibitor of ACh release (Landau & Nachsen, 1975; E.M.S. unpublished). As matching levels of ACh secretion were employed to calculate affinity and efficacy, the quantitative results were not dependent upon pH (unpublished). Solutions were delivered at a constant rate by means of a roller pump (Spectroderm). Experiments were carried out at room temperature.

Tubocurarine chloride and 2-chloroadenosine were obtained from Sigma Chemical Co., St. Louis, Missouri. An anti-cholinesterase was *not* employed in this study.

Determination of the number of acetylcholine quanta released synchronously by a nerve impulse ($\bar{m}$)

Under conditions in which $\overline{m.e.p.ps}$ could be recorded directly, $\bar{m}$ was determined from the ratio of the mean computer-averaged, endplate potential amplitude ($\overline{e.p.p.}$) to the mean $\overline{m.e.p.p.}$ amplitude ($\overline{m.e.p.p.}$) (Del Castillo & Katz, 1954) using appropriate numbers of $\overline{e.p.ps}$ (400–800/m) (see Rahamimoff, 1967). In experiments in which the $\overline{e.p.p.}$ was <5 mV, no correction was made for non-linear summation (Martin, 1955) at normal resting potentials (80–100 mV). E.p.ps >5 mV, were corrected using the mean of Martin's original correction (1955) (which tends to overcorrect, Martin, 1976) and Stevens (1976) correction (which undercorrects). At low levels of ACh release (e.g., after La^{3+} treatment), the method of failures (del Castillo & Katz, 1954) was employed as well.

When tubocurarine chloride (Tc) was used to paralyse neuromuscular transmission, $\bar{m}$ was calculated from the following equation (Ceccarelli & Hurlbut, 1975):

$$\bar{m} = \frac{\overline{e.p.p_c}}{\overline{m.e.p.p.}} (1 + 4[Tc]) \qquad (2)$$

where $\overline{e.p.p._c}$ is the $\overline{e.p.p.}$ in Tc corrected for non-linear summation, [Tc] is in mg/litre and 4 represents the affinity constant (litre/mg) of Tc for the ACh receptor. The m.e.p.p. was determined in Tc-free Ringer. The use of this equation will be justified in the following section.

Construction of log concentration-$\bar{m}$ curves to saturation for Ca and Sr

M.e.p.ps were measured in various Ca and Sr concentrations before addition of Tc. Preparations were then curarized with [Tc] varying from 6–10 mg/litre (high [Tc]) in a [Ca] that produced maximal responses (5.4–7.2 mM). [Tc]s of 6–8 mg/litre were used most frequently because these concentrations (Auerbach & Betz, 1971) do not appear to have presynaptic effects at frog neuromuscular junctions yet ensured that e.p.ps were subthreshold for generation of muscle action potentials in high calcium concentrations. In high [Tc] solutions e.p.ps were measured in [Ca] ranging from 7.2 mM–0.6 mM and $\bar{m}$ calculated using eqn.(2). Preparations were then washed with 0.25–0.9 mM Ca Ringer in 1.0–2.5 mg/litre Tc (low [Tc]) to construct the lower end of the log [Ca]-$\bar{m}$ relation. The complete log [Sr]-$\bar{m}$ curve was determined entirely in low [Tc]. When e.p.ps in Tc were recorded at a different resting potential from m.e.p.ps in Tc-free solution, appropriate corrections for changes in resting potential were made.

During most experiments, the applicability of equation (2) was tested in different [Tc], [Ca] and [Sr] as follows. At some time during the experiment, $\bar{m}$ was measured directly in Tc-free solution (using 0.3–0.4 mM Ca or 1–2 mM Sr). The preparation was then curarized (1–2 mg/litre Tc) and $\bar{m}$ calculated using equation (2). Calculated $\bar{m}$ values were also compared in 0.9 mM Ca at high and low [Tc]. The results were discarded when $\bar{m}$ deviated by more than 10% between different [Tc] and unparalysed preparations.

A seemingly anomalous but correctable phenomenon occurred in all experiments in high [Tc] and [Ca]s which produced elevated levels of ACh release. After apparent equilibration in 5.4–7.2 mM Ca (8 mg/litre Tc), e.p.ps in response to repetitive nerve stimulation even at frequencies as low as 0.01 Hz declined and eventually reached a new stable level. Washing into Tc-free Ringer revealed that the m.e.p.p. amplitude was reduced irreversibly by approximately the same fraction as the corrected e.p.p. in Tc. Experimental results were corrected for this effect by measuring $\overline{m.e.p.p.}$ both before and after curarization two to three times during the course of experiments in which concentration-$\bar{m}$ curves were constructed for Ca and Sr in the same fibre. The $\bar{m}$

values were then corrected to the initial value of $\bar{m}$ produced by the first several stimuli in high Ca, high Tc. The cause of the stimulation-dependent depression of the e.p.p. is unknown. The parallel decline in e.p.p. and m.e.p.p. amplitudes, however, suggest that it may be related to the blockade of open endplate channels by Tc (Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979).

Although the decay in e.p.p. and m.e.p.p. was irreversible at Tc concentrations $\geq$ 8 mg/litre, the effect appeared reversible at lower Tc concentrations.

Theoretical basis for determining affinity (K) and efficacy (e) of Ca and Sr

Several excellent reviews concerning the mathematical framework of receptor theory have been published (e.g. Waud, 1968; Stephenson & Barlow, 1970; Rang, 1971; Colquhoun, 1973, 1979; Thron, 1973). For the sake of simplicity, the interaction of Ca and Sr with the hypothetical presynaptic X site will be presented here in accordance with the modified occupation theory (Stephenson, 1956). The derivations of eqns (3)–(9) all utilize *null* methods in which equal levels of the ACh secretion (equal $\bar{m}$) are compared. This eliminates assumptions as to the shape of the relationship between $\bar{m}$ and [Ca] and [Sr]. It is further assumed that equal $\bar{m}$ values reflect equal products of efficacy and occupancy. Stephenson (1956) called this product the stimulus (S), that is, $S_{Me} = y_{Me}$; note that S is assumed to be related linearly to both e and y. This implies that Me binding follows a simple rectangular hyperbola, an assumption that is in accord with receptor binding studies (see Colquhoun, 1973) and studies on Ca fluxes (see Discussion). Implicit in this interpretation is that the non-linearity in the [Me]-$\bar{m}$ relation must be due to events subsequent to e and y, (i.e., due to the non-linear relationship between S and ACh secretion). Furthermore, as the results suggest that y reflects Ca channel occupancy and e may represent an intraterminal Ca affinity (see Discussion), it seems plausible to assume that steps subsequent to e and y are independent of changes in extracellular [Me] in the range employed here for quantitation (see e.g. Katz & Miledi, 1967; Katz, 1969).

(1) *Determination of* K_{Sr} (a) *Comparison of equal* $\bar{m}$ *produced in Ca and in Sr solutions* (Stephenson & Barlow, 1970; Colquhoun, 1973). On the assumption that equal $\bar{m}$ reflect equal stimuli,

$$S_{Ca} = S_{Sr}$$

$$e_{Ca} y_{Ca} = e_{Sr} y_{Sr}$$

Substituting for y in equation (1).

$$e_{Ca} \frac{K_{Ca}[Ca]}{1 + K_{Ca}[Ca]} = e_{Sr} \frac{K_{Sr}[Sr]}{1 + K_{Sr}[Sr]}$$

Ca(high e) is used in low concentration compared to Sr(low e) to produce equal $\bar{m}$. If e_{Ca} is sufficiently large and K_{Ca} is very low (see Figure 9) then spare receptors will exist. Thus

$$[Ca]\,K_{Ca} \ll 1$$
$$\text{and } S_{Ca} = e_{Ca}\,K_{Ca}\,[Ca] \qquad (3)$$

The resulting expression for matching $\bar{m}$ is

$$e_{Ca} K_{Ca}[Ca] = e_{Sr} \frac{K_{Sr}[Sr]}{1 + K_{Sr}[Sr]}$$

which can be rearranged to give

$$\frac{1}{[Ca]} = \frac{e_{Ca}K_{Ca}}{e_{Sr}K_{Sr}} \frac{1}{[Sr]} + \frac{e_{Ca}K_{Ca}}{e_{Sr}} \qquad (4)$$

As (4) is a straight line, a plot of $[Ca]^{-1}$ that produces equal $\bar{m}$ with $[Sr]^{-1}$ yields a straight line with slope = $e_{Ca}K_{Ca}/e_{Sr}K_{Sr}$ and intercept = $e_{Ca}K_{Ca}/e_{Sr}$. K_{Sr} can thus be estimated from the intersection of the line with the abscissa (at $[Ca]^{-1} = 0$, $[Sr]^{-1} = -K_{Sr}$) or from the ratio of the intercept to the slope.

(b) *Comparison of equal* $\bar{m}$ *produced in Ca solutions in the presence and absence of Sr* (Stephenson, 1956; Colquhoun, 1973). If $[Ca]_{Sr}$ is the concentration of calcium that produces equal $\bar{m}$ to that produced by a concentration of calcium [Ca] in the absence of Sr then equal stimuli are described by

$$e_{Ca} \frac{K_{Ca}[Ca]}{1 + K_{Ca}[Ca]} = \frac{e_{Ca}K_{Ca}[Ca]_{Sr} + e_{Sr}K_{Sr}[Sr]}{1 + K_{Ca}[Ca]_{Sr} + K_{Sr}[Sr]}$$

Rearranging and assuming $e_{Ca} \gg e_{Sr}$ (i.e., spare receptors)

$$[Ca] = [Ca]_{Sr} \frac{1}{K_{Sr}[Sr] + 1} + \frac{e_{Sr}[Sr]}{K_{Ca}} \qquad (5a)$$

Eqn. (5a) again describes a straight line. By plotting [Ca] against $[Ca]_{Sr}$, K_{Sr} can be estimated from the slope of the line

$$K_{Sr} = \frac{(1/\text{slope}) - 1}{[Sr]} \qquad (5b)$$

(c) *Sr as a competitive antagonist of Ca.* The partial agonist, Sr acts as a competitive inhibitor of Ca when the effects of Sr in supporting ACh release are eliminated by first treating with an irreversible 'competitive' antagonist (Furchgott & Bursztyn, 1968). Equating stimuli:

$$\frac{e_{Ca}K_{Ca}[Ca]}{1+K_{Ca}[Ca]} = \frac{e_{Ca}K_{Ca}[Ca]_{Sr}}{1+K_{Ca}[Ca]_{Sr}+K_{Sr}[Sr]}$$

$$\frac{[Ca]_{Sr}}{Ca} - 1 = K_{Sr}[Sr] \text{ or}$$

$$K_{Sr} = (\text{dose/ratio} - 1)/[Sr] \quad (6)$$

where $[Ca]_{Sr}/[Ca]$ is defined as the dose-ratio (Gaddum, 1926), Eqn. (6) is generally termed the Schild equation (Schild, 1947; Arunlakshana & Schild, 1959). K_{Sr} can be estimated from dose-ratios or from the intercept of a plot of log (dose-ratio − 1) against log [Sr]. This equation does not require the spare receptor assumption in its derivation and is the most useful method for measuring affinity constants of antagonists in complex biological systems (see e.g. Stephenson & Barlow, 1970). Note that eqn. (5) reduces to eqn. (6) when $e_{Sr} \rightarrow 0$ (i.e., if Sr is a pure antagonist).

(2) *Determination of* K_{Ca} Furchgott, 1966; Waud, 1968; Stephenson & Barlow, 1970; Colquhoun 1973). If $[Ca]_{irr}$ is the concentration of Ca that after occlusion of a fraction of receptors z by an irreversible competitive antagonist produces equal $\bar{m}$ to a concentration of Ca,[Ca], in the absence of an irreversible antagonist, then equal stimuli produce

$$\frac{e_{Ca}K_{Ca}[Ca]}{1+K_{Ca}[Ca]} = \frac{e_{Ca}(1-z)K_{Ca}[Ca]_{irr}}{1+K_{Ca}[Ca]_{irr}}$$

into which upon rearrangement gives the equation of straight line

$$\frac{1}{[Ca]} = \frac{1}{1-z} \cdot \frac{1}{[Ca]_{irr}} + \frac{zK_{Ca}}{1-z} \quad (7a)$$

Plotting [Ca] agonist $[Ca]_{irr}$ and estimating z from the slope

$$z = \frac{\text{Slope} - 1}{\text{Slope}} \quad (7b)$$

K_{Ca} was estimated by extrapolation as when $[Ca]^{-1} = 0$, $[Ca]^{-1}_{irr} = -zK_{Ca}$

(3) *Determination of* e_{Ca} If $S = 1$ is arbitrarily defined as the calcium concentration that produces a half-maximal response, i.e. the $[Ca]_{50}$ (Stephenson, 1956) then from eqn. (3)

$$1 = e_{Ca}K_{Ca}[Ca]_{50} \quad (8a)$$

as $[Ca]_{50}$ can be determined from log [Ca] - $\bar{m}$ curves and K_{Ca} can be determined as above, e_{Ca} can then be calculated by:

$$e_{Ca} = (K_{Ca}[Ca]_{50})^{-1} \quad (8b)$$

(4) *Determination of* e_{Sr} For e_{Sr}, first $[Ca]_{50}$ is estimated; then $e_{Ca}K_{Ca}$ determined from eqn. (8a). As the intercept in eqn. (4) $= \frac{e_{Ca}K_{Ca}}{e_{Sr}}$,

$$e_{Sr} = \frac{1}{(\text{intercept})([Ca]_{50})} \quad (9)$$

Results

General observations on concentration-effect relationships

It appears of interest to begin this study by comparing log [Me]-$\bar{m}$ curves constructed for Ca and Sr to saturation. If Ca and Sr have the same K_{Me} (Meiri & Rahamimoff, 1971), then, by the mass action formulation, both Me substrates should produce half-maximal responses at the same concentration, (the $[Me]_{50}$, see Stephenson, 1956). Figure 1 illustrates typical log [Me]-$\bar{m}$ relationships. In Ca solutions (circles) the maximum $\bar{m}$ (m_{max}) = 1008 is achieved at a [Ca]≤7.2 mM with the $[Ca]_{50}$ = 2.7 mM (arrow). In

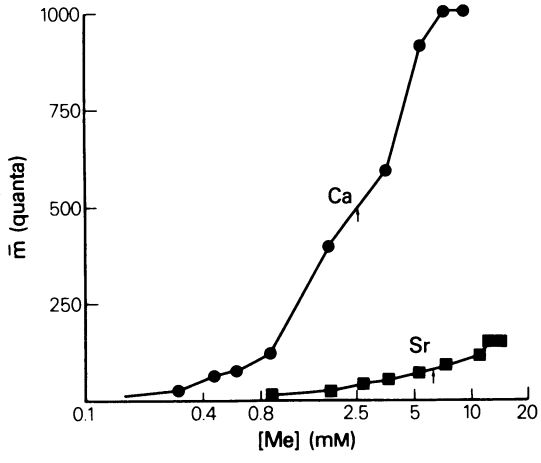

Figure 1 A comparison of the log concentration-effect relationships for the divalent metal (Me) agonists Ca and Sr in supporting evoked acetylcholine (ACh) release. In this experiment, the curve of log [Ca] plotted against the mean number of ACh quanta released by a nerve impulse ($\bar{m}$) (●) has a maximum $\bar{m}$ ($\bar{m}_{max}$) of 1008 achieved at a [Ca]≤ 7.2 mM. The concentration of Me that produces a half-maximal response ($[Me]_{50}$) for Ca= 2.7 mM. For the log [Sr] - $\bar{m}$ relation in this same fibre (■), m_{max} = 150 at a [Sr] ≤ 12.5 mM with the $[Sr]_{50}$ = 6.2 mM. Compared to Ca, the log [Me] - $\bar{m}$ value for Sr is thus shifted to the right as well as having a smaller $\bar{m}_{max}$. This experiment represents log[Me] - $\bar{m}$ curves 1A and 1B in Table 1.

Table 1 A summary of log [Me]-m curves for Ca and Sr

Experiment	*Me agonist*	m_{max} (quanta)	$[Me]_{50}$ (mM)
1A	Ca	1008	2.7
2A	Ca	1250	3.1
3A	Ca	274	3.0
4	Ca	2600	2.1
5	Ca	993	2.8
6	Ca	850	3.1
7	Ca	600	2.4
8	Ca	112	2.0
		961 ± 270	2.6 ± 0.2 (mean + 1 s.e.mean, $n = 8$)
1B	Sr	150	6.2
2B	Sr	240	8.1
3B	Sr	92	6.2
4	Sr	120	6.8
5	Sr	84	6.2
6	Sr	38	3.6
7	Sr	37	7.6
		109 ± 27	6.4 ± 0.5 (mean ± 1 s.e.mean, $n = 7$)

For all log [Ca]-m curves, m_{max} was reached between 5.4 and 7.2 mM Ca. For Sr, m_{max} was reached between 9.0 and 13.5 mM Sr. Experiments 1, 2, 3 are those in which log[Ca]-$\overline{m}$ curves (A) and log[Sr]-$\overline{m}$ curves (B) are from the same fibre. For further details see text.

Sr solutions (squares) the $\overline{m}_{max}$ in the same fibre is 150, a value attained at a [Sr] of 12.5 mM, with the $[Sr]_{50}$ = 6.2 mM (arrow). It thus appears that the log [Sr]-m curve, in addition to having a smaller $\overline{m}_{max}$ than Ca, is shifted to the right as well. Similar results were observed in all other experiments (see Table 1) with the mean $[Sr]_{50}$ = 6.4 ± 0.5 mM (mean ± 1 s.e.mean, $n = 7$) ≅ 2.4 times the mean $[Ca]_{50}$ = 2.6 ± 0.2 mM(mean ± s.e.mean, $n = 8$).

The differences in $\overline{m}$ between Ca and Sr are unlikely to be produced by differences in the amplitude of nerve terminal action potentials (n.t.ps). In the experiment shown in Figure 2, no differences or computer averaged n.t.p. amplitude were observed in 1.8 mM Sr (a), 1.8 mM Ca (b), 9.0 mM Sr (c) and 7.2 mM Ca Ringer (d). This is in contrast to the differences in focal endplate currents, (e.p.cs) which roughly reflect ACh secretion in (a)–(d) (see Figure legend). As $\overline{m}_{max}$ was produced at 9.0 mM Sr and ≤ 7.2 mM Ca in this fibre, it seems that the differential abilities of Ca and Sr to support ACh release are not due to differences in the level of membrane depolarization associated with the nerve terminal action potential. It thus appears that the log [Me]-$\overline{m}$ relations illustrated in Figure 1 reflect the agonist actions of Ca and Sr in the process of depolarization-secretion coupling. The observed shift to the right of the $[Sr]_{50}$ as compared to $[Ca]_{50}$ (Figure 1 and Table 1) is incompatible with Ca and Sr having equal affinity (Meiri & Rahamimoff,

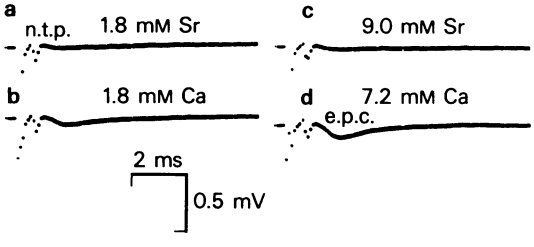

Figure 2 Nerve terminal action potentials (n.t.ps) in 1.8 mM Sr(a), 1.8 mM Ca(b), 9.0 mM Sr(c) and 7.2 mM Ca(d). The Ringer solution contained 8 mg/litre tubocurarine. Each trace is the computer-averaged response to 128 nerve stimuli (0.3 Hz). Note that the n.t.p. amplitudes in (a)–(d) are essentially indistinguishable. In this fibre 9.0 mM Sr(c) produced $\overline{m}_{max}$ and 7.2 mM Ca was > [Ca] needed for $\overline{m}_{max}$ (d). Focal endplate current (e.p.c.) amplitudes do not accurately reflect the large differences in $\overline{m}$ between Sr and Ca as the response to an individual ACh quantum is somewhat larger in Sr than in Ca.

1971) and/or is incompatible with the simple mass action assumption for Ca (Stephenson, 1956).

The effects of irreversible antagonists of evoked acetylcholine release on $\bar{m}$ in Ca and Sr solutions: qualitative evidence for spare Ca receptors

The results in the preceding section suggest that it may be profitable to evaluate the simple mass action assumption by testing for the presence of spare presynaptic Ca receptors (Stephenson, 1956). To apply this test, it is necessary to select an irreversible antagonist that competes, at least during its initial exposure, with the agonist at the receptor site under investigation. If, in the presence of an irreversible occlusion of a proportion of the receptor population, increasing the concentration of the agonist allows the antagonism to be overcome, then the presence of spare receptors is suggested (see e.g. Waud, 1968). As the site responsible for coupling depolarization to secretion is normally activated from the external surface of the nerve ending and is associated with Ca (and presumably Sr) fluxes (Katz, 1969), it appears that agents which act extracellularly both to block Ca fluxes in nerve endings and to depress $\bar{m}$ irreversibly would serve as appropriate tools to test for spare Ca receptors.

Effects of La^{3+}. La^{3+} has been shown to block regenerative Ca currents in nerve endings (Miledi, 1971; Reuter, 1973) and to depress $\bar{m}$, possibly irreversibly (Heuser & Miledi, 1971; de Bassio, Schnitzler & Parsons, 1971). The experiment of Figure 3 illustrates the irreversibility of La^{3+} antagonism. In this experiment, the initial $\bar{m}$ was 27 in a control Ringer solution of 0.6 mM Ca (filled circles). The addition of 1 μM La to the control Ringer (between arrows, open circles) caused $\bar{m}$ to fall during the period of La^{3+} application and to continue to fall after La^{3+} was removed from the bathing fluid. This effect of La^{3+} was irreversible, even after 45 min in La^{3+}-free solution, with the final level of $\bar{m}$ = 4. Despite the irreversibility, however, doubling the [Ca] to 1.2 mM (in the presence of 1 μM La^{3+}, concentric circles) overcame the antagonism and, after approximately 4 min, produced suprathreshold e.p.ps (due to postsynaptic effects of La^{3+}) with $\bar{m}$ = 20. The preparation was then rapidly superfused with 0.6 mM Ca Ringer without reaching a final $\bar{m}$ in 1.2 mM Ca + 1 μM La^{3+} Ringer to prevent the microelectrode being dislodged by muscle twitches. Within 3 min after returning to 0.6 mM Ca Ringer, $\bar{m}$ = 4 was restored suggesting that the ability of elevated Ca to surmount the irreversible La^{3+} antagonism was not due to a displacement by Ca of bound La^{3+}. Finally, a 200 μM increase in the [Ca] in the bathing fluid (from 0.6–0.8 mM, squares) caused $\bar{m}$ to increase to 15 (in the presence or absence of La^{3+}). In other experiments in which partial log [Ca]-$\bar{m}$ curves were constructed (Figure 4a), it can be shown that the control log [Ca]-$\bar{m}$ curve (Figure 4a, circles) is shifted to the right in an apparently parallel fashion by La^{3+} (2 μM, squares).

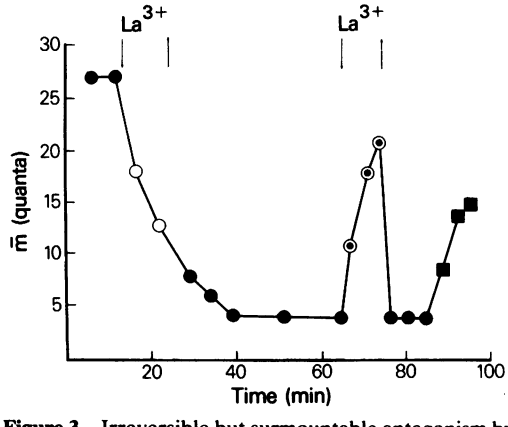

Figure 3 Irreversible but surmountable antagonism by La^{3+} (1 μM) of the effects of Ca. In the control solution of 0.6 mM Ca Ringer (●), $\bar{m}$ = 27. Superfusion with 0.6 mM Ca + 1 μM La^{3+} Ringer (○) $\bar{m}$ declined and continued to fall after removal of La^{3+}. Even after 45 min in La^{3+}-free Ringer, $\bar{m}$ remained depressed at a steady level of 4. Despite this irreversible antagonism, washing with 1.2 mM Ca + 1 μM La^{3+} Ringer increased $\bar{m}$ to 20 (⊙) and e.p.ps became supra-threshold. Changing the solution back to 0.6 mM Ca returned $\bar{m}$ = 4. Finally, bathing in 0.8 mM Ca Ringer (■) (in the presence or absence of La $^{3+}$) elevated $\bar{m}$ to 15. Each point is the corrected computer-averaged $\overline{e.p.p.}$ produced in response to 32 nerve stimuli (0.5 Hz) divided by $\overline{m.e.p.p.}$

A plausible explanation of these results is that irreversible antagonism by La^{3+} can be surmounted by increasing [Ca] because Ca can 'mop-up' spare binding sites not occupied by La^{3+}. If this explanation is valid, then the relationship between Sr and La^{3+} should differ markedly from that between Ca and La^{3+}. Sr acts as a partial agonist (Meiri & Rahamimoff, 1971) and needs to occupy a substantial proportion of the binding sites in order to produce even moderate-sized responses; antagonism of Sr by La^{3+} should thus be insurmountable. Figure 4b shows this to be the case. In this experiment, the control [Sr]-$\bar{m}$ curve is not restored after irreversible La^{3+} antagonism (1 μM) by increasing [Sr] (squares). In fact, in this experiment and in two other experiments it was impossible to restore multiquantal evoked ACh secretion by increasing [Sr] in the presence of 1–2 μM La^{3+} (i.e., the maximum averaged e.p.p. consisted of a m.e.p.p. phase-locked to the nerve impulse). Although in other experiments $\bar{m}$ could be restored to $\leq$ after La^{3+} antagonism by increasing [Sr], there was never an indication of a parallel shift in the log [Sr]-$\bar{m}$ relations as was observed in all four experiments done with Ca at a similar level of ACh release and 0.5–2 μM La^{3+}.

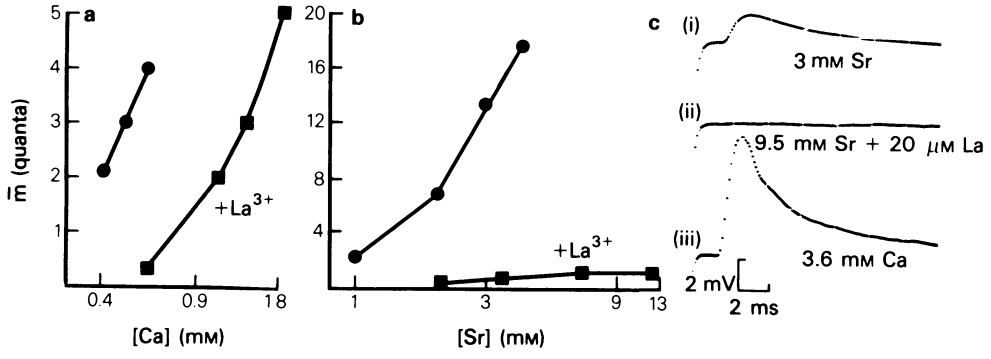

Figure 4 A comparison of the Ca-La^{3+} relationship with that for Sr-La^{3+}. Partial log [Me] -$\bar{m}$ curves for Ca (a) and Sr(b) before (●) and after (■) treatment with La^{3+}. In (a) note the apparent parallel shift in the log [Ca]-$\bar{m}$ relation after irreversible occlusion of the response with La^{3+} (2 μM). In (b) the log [Sr]-$\bar{m}$ relation in this fibre was not shifted in parallel after irreversible occlusion with La^{3+} (1 μM). The results are consistent with the presence of spare receptors for Ca, but not for Sr. In these studies, after La^{3+} treatment, responses were inhibited to a steady-state level by washing into La^{3+}-free solution after La^{3+} treatment in most fibres. This served to make for more reproducible log [Me]-$\bar{m}$ curves. As La^{3+} appears to be an irreversible 'competitive' inhibitor of Ca, under appropriate conditions (high concentration or sufficient time) La^{3+} will eliminate all responses to Ca (see Heuser & Miledi, 1971). (c) Selective elimination of Sr-dependent ACh release in 20 μM La^{3+}: (i) e.p.p. in 3 mM Sr, $\bar{m}$=2.3; in 3 mM Sr + 20 μM La^{3+} (not shown) and in 9.5 mM Sr + 20 μM La^{3+} m~0 (ii). (iii) $\overline{e.p.p.}$ 3 min after washing with 3.6 mM Ca Ringer, m=14. Note that 3 min after (iii), $\bar{m}$ increased to ~40 and the $\overline{e.p.p.}$ in this fibre became suprathreshold. In several fibres in this preparation, suprathreshold $\overline{e.p.p.s}$ and muscle twitches occurred during the determination of $\overline{e.p.p.}$ shown in (iii). Each $\overline{e.p.p.}$ is the average of 32 stimuli (0.3 Hz). In a total of 4 experiments, 20–120 μM La^{3+}, after an initial phase of increased release (see Alnaes & Rahamimoff, 1974), totally eliminated all evoked ACh release in 10 mM Sr Ringer at a time when large responses were observable in 1.8–3.6 mM Ca Ringer. These results suggest that increases in [Me] do not act nonselectively to increase the driving force through unblocked receptors; rather, Ca can 'mop-up' spare receptors selectively.

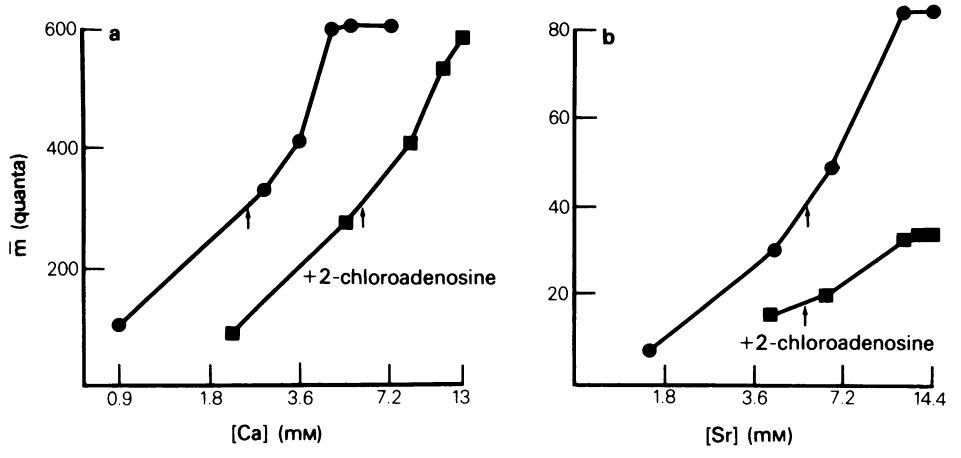

Figure 5 Log [Me]-$\bar{m}$ curves for Ca (a) and Sr (b) before (●) and after (■) irreversible antagonism with 2-chloradenosine (25 μM). Note the apparent competitive relationship between Ca and 2-chloradenosine and the non-competitive relationship between Sr and 2-chloradenosine. Arrows show half maximal responses. For further details, see text.

Further evidence for the presence of spare Ca receptors was obtained with higher $[La^{3+}]$s. In the experiment shown in Figure 4c, the control e.p.p. in 3 mM Sr (m = 2.3 (i)) is reduced to ~0 by 20 μM La^{3+}, an antagonism which was not overcome by raising the [Sr] to 9.5 mM (ii) in the presence of 20 μM La^{3+}. In contrast, 3 min after substituting with 3.6 mM Ca, $\bar{m}$ rose to 14 (iii) and after another 4 min, $\bar{m}$ increased to 40 and the e.p.p. became suprathreshold (not shown). The experiment in Figure 4c indicates that responses mediated by Sr^{2+} can be eliminated entirely by La^{3+}, yet leave large responses to Ca.

In experiments with higher $[La^{3+}]$ (>10 μM), $\bar{m}$ often increased substantially (cf. Alnaes & Rahamimoff, 1974) before falling to very low levels. Furthermore, in all experiments, the m.e.p.p. frequency increased in the presence of La^{3+}, an effect which was quite dramatic at higher $[La^{3+}]$ (see Heuser & Miledi, 1971). Using La^{3+}-containing liposomes to apply La^{3+} intracellularly, it can be shown that these effects of La^{3+} in increasing ACh secretion occur at an intraterminal locus and appear to be relatively independent of the extracellular Ca-La^{3+} antagonism (Kharasch, Mellow & Silinsky, 1981). The results suggest that La^{3+} is an irreversible 'competitive' antagonist of Ca, although the secondary effects of La^{3+} (increases in m.e.p.p. frequency and changes in postsynaptic sensitivity) make it impractical technically to generate reliable curves of log [Me]-$\bar{m}$ to $\bar{m}_{max}$ in curarized preparations treated with La^{3+}.

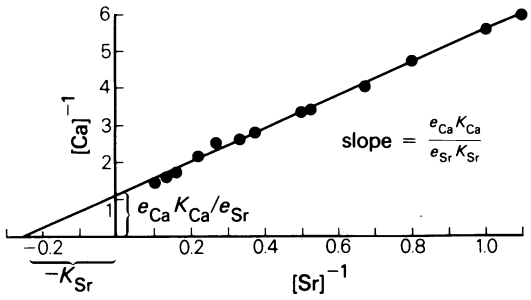

Figure 6 Calculation of K_{Sr} by comparing $[Ca]^{-1}$ with $[Sr]^{-1}$ that produces matching $\bar{m}$. Note that the experimental points fall on a straight line, as would be predicted by eqn (4) which was derived using the spare receptor assumption. The values of the slope, intercept and K_{Sr} (eqn. 4) are indicated on the graph, which was constructed from the experiments of Figure 1. K_{Sr} in this experiment is 0.25 mM^{-1}. The mean K_{Sr} from four experiments using eqn (4) was 0.26±0.03 mM (mean ± s.e. mean). To construct these curves, points on the log [Sr]-$\bar{m}$ curve were chosen at convenient intervals in the range between 0.9 and 9.0 mM Sr and then matched to the [Ca] that produces equal $\bar{m}$. This particular range of Sr was employed as at higher [Sr], non-selective surface charge effects of Sr may influence nerve excitability (see Discussion) and/or b) at [Ca] that produce equal $\bar{m}$ to [Sr]> 9 mM, the spare receptor assumption may become invalid. The points were fitted by eye to a straight line. For further details, see text.

Effects of 2-chloroadenosine Adenosine compounds have been shown recently to depress the entry of Ca into depolarized nerve endings (Ribeiro, Sa'-Almeida & Namorado, 1979). Furthermore, it has been demonstrated that the depressant effects of adenosine derivatives on evoked ACh release (Ginsborg & Hirst, 1972) are mediated by specific adenosine receptor (Silinsky, 1980b) sites that apparently are situated at the external surface of nerve endings (R-sites, requiring an intact ribose moiety, see London & Wolff, 1977). 2-Chloroadenosine is an R-site agonist which reduces $\bar{m}$ (Silinsky, 1980b) irreversibly at a concentration of 25 μM (e.g. Figure 5). At this concentration antagonism occurs rapidly (maximum inhibition being achieved within 2 min) and without concomitant changes in m.e.p.p. frequency or postsynaptic sensitivity to ACh. These features suggested that it would be of interest to examine the effects of irreversible antagonism of evoked ACh release by 2-chloroadenosine (25 μM) on the log [Ca] and [Sr]-$\bar{m}$ curves constructed to the level of $\bar{m}_{max}$. Figure 5a shows an experiment in which the control log [Ca]-$\bar{m}$ curve (circles, $[Ca]_{50}$ = 2.4 mM) was shifted to the right by 2-chloroadenosine (squares, $[Ca]_{50}$ = 5.7 mM) without a decrease in $\bar{m}_{max}$. In two other experiments, $[Ca]_{50}$ was shifted from 3.0 mM to 5.4 mM and from 2.0 mM to 4.6 mM by 25 μM 2-chloroadenosine. In contrast, $\bar{m}_{max}$ for the log [Sr]-$\bar{m}$ relation (Figure 5b) filled circles, $[Sr]_{50}$ = 6.0 mM) was depressed to less than half by 2-chloroadenosine without a change in the $[Sr]_{50}$ (filled squares, $[Sr]_{50}$ = 5.9 mM). Indeed in all 3 experiments, the control $[Sr]_{50}$ (6.8 ± 0.4 mM M^{-1}, n = 3) was indistinguishable from the $[Sr]_{50}$ after treatment with 25 μM 2-chloroadenosine (6.9 ± 0.4 mM, n = 3) although the $\bar{m}_{max}$ could only be restored to a mean of ~40% of the control value in the 3 experiments. In the 3 experiments with Ca, the greater part of the log [Ca]-$\bar{m}$ relation showed a parallel shift in the presence of 2-chloroadenosine.

In experiments other than that shown in Figure 5a, $\bar{m}_{max}$ for Ca could be restored only to 80% of the pre-antagonized level, an effect that might be due to non-selective effects of high [Me] on n.t.ps (see Discussion).

The apparent competitive relationship between 2-chloroadenosine and Ca (Figure 5a) and the non-competitive relationship between this irreversible antagonist and Sr (Figure 5b) in conjunction with the results with La^{3+}, suggest qualitatively that Ca produces maximal levels of ACh release whilst leaving a proportion of Ca binding sites spare. In view of the failure of the simple mass action formulation to predict the effects of irreversible antagonists, it appears

necessary to re-evaluate the affinity and efficacy of Ca and Sr within the mathematical framework of the receptor theory.

Determination of K_{Sr}: quantitative evidence for spare Ca receptors

If the hypothetical membrane site X is envisaged as a receptor, then there are a variety of arithmetical devices at the disposal of the investigator to determine K_{Sr}. As some of these approaches require the spare receptor assumption for equations to be transcribed into useful forms, whilst others can be derived independently of such an assumption (see Methods), agreement in K_{Sr} values between the various methods would suggest quantitatively that spare Ca receptors are present at presynaptic sites.

The first method compares equal $\bar{m}$ in Ca and in Sr solutions and uses the spare receptor assumption to obtain eqn. (4) (e.g., Stephenson & Barlow, 1970; see Methods for derivation and complete references for all equations).

$$\frac{1}{[Ca]} = \frac{e_{Ca}K_{Ca}}{e_{Sr}K_{Sr}} \cdot \frac{1}{[Sr]} + \frac{e_{Ca}K_{Ca}}{e_{Sr}} \qquad (4)$$

Figure 6 shows a typical experimental result in which $[Ca]^{-1}$ is plotted against the $[Sr]^{-1}$ that produced a matching $\bar{m}$. Extrapolating the straight line to $[Ca]^{-1} = 0$ produces $K_{Sr} = 0.25$ mM^{-1}. The mean K_{Sr} in all such experiments was $K_{Sr} = 0.26 \pm 0.03$ mM ($\pm$ 1 s.e.mean, $n = 4$).

In the second method for calculating K_{Sr} (assuming spare receptors), log [Ca]-$\bar{m}$ curves were constructed (Figure 7a) in the presence (filled squares) and absence (filled circles) of Sr (2 mM) and [Ca] was plotted against the Ca concentration that produced a matching $\bar{m}$ in the presence of Sr ($[Ca]_{Sr}$), Figure 7b). According to drug-receptor theory (Colquhoun, 1973) the relationship between [Ca] and $[Ca]_{Sr}$ should be of the form

$$[Ca] = \frac{[Ca]_{Sr}}{K_{Sr}[Sr] + 1} + \frac{e_{Sr}[Sr]}{K_{Ca}} \qquad (5a)$$

which predicts a straight line. Figure 7b shows the results from Figure 7a plotted in this form. K_{Sr} was estimated from

$$K_{Sr} = \frac{(1/\text{Slope})\text{-}1}{[Sr]} \qquad (5b)$$

In the experiment illustrated in Figure 7, $K_{Sr} = 0.25$ mM^{-1} and in all experiments, $K_{Sr} = 0.24 \pm 0.01$ (mean$\pm$ s.e.mean, $n = 3$). It thus appears that both methods which involve the spare receptor assumption

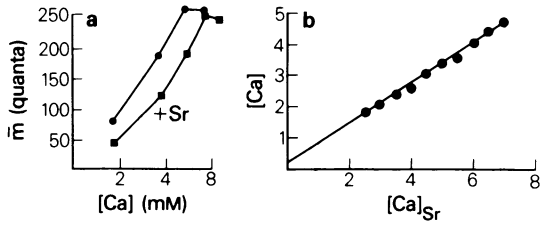

Figure 7 Calculation of K_{Sr} by comparing Ca concentrations that produce matching $\bar{m}$ in the presence ($[Ca]_{Sr}$ and absence ([Ca]) of Sr. The experimental results are shown in (a). In (b), the results from (a) are plotted in accordance with eqn (5a), which was derived using the spare receptor assumption. Note that the points fall on a straight line which was drawn by eye through matching $\bar{m}$ chosen at ~0.5 mM intervals, on the Ca_{Sr} curve. $K_{Sr} = 0.25$ mM^{-1} as calculated by eqn (5b). The mean K_{Sr} calculated by this method was 0.24 ± 0.01 mM^{-1} (mean $\pm$ s.e. mean, n=3).

produced statistically indistinguishable values for K_{Sr} (e.g., Student's t test).

As the crucial test of the spare receptor assumption it is necessary to calculate K_{Sr} using Sr as a competitive inhibitor of Ca. This method has much theoreti-

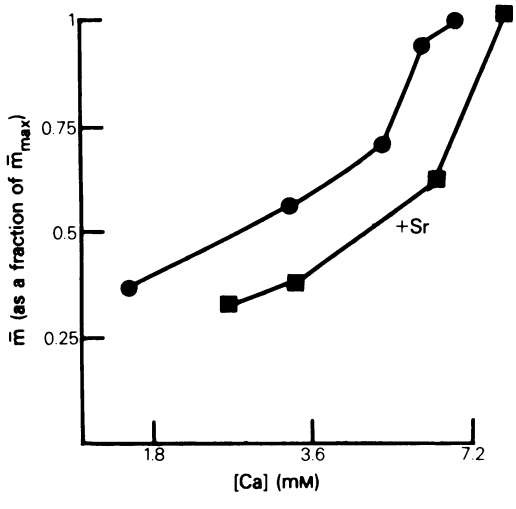

Figure 8 Log [Ca]-$\bar{m}$ curves in the absence (●) and presence (■) of the competitive inhibitor, Sr (2 mM). The preparation was treated with 1 μM La^{3+} for 14 min to eliminate $\bar{m}$ in 2 mM Sr. This, in effect, converted Sr from a partial agonist to a competitive inhibitor as can be seen from the similar dose-ratios at all levels of the curve. K_{Sr} calculated by comparing matching $\bar{m}$ at three different [Ca] ranged from 0.33–0.36 mM^{-1}. For all experiments the K_{Sr} as a competitive inhibitor was 0.27 ± 0.02 mM^{-1} ($n = 6$). For further details see text.

cal justification (see e.g., Stephenson & Barlow, 1970; Colquhoun, 1973) and, as described below, is valid whether or not spare receptors are normally present for Ca. In the experiment shown in Figure 8, the effect of 2 mM Sr in supporting ACh release was eliminated by 13 min of exposure to 1 μM La^{3+}. The log [Ca]-$\bar{m}$ curves were then constructed for Ca in the absence (Figure 8, circles) and presence (squares) of Sr ($[Ca]_{Sr}$). Using the Schild eqn.

$$K_{Sr} = (\text{dose-ratio} - 1)/[Sr] \qquad (6)$$

where $[Ca]_{Sr}/[Ca]$ is the dose-ratio (the ratio of the concentration of Ca that produce the same $\bar{m}$ in the presence and absence of Sr). K_{Sr} can be calculated at different [Ca]s and in this experiment, K_{Sr} ranged from 0.33 to 0.36 at various matching $\bar{m}$'s. In five other experiments, K_{Sr} calculated by eqn. (6) varied from 0.20–0.29 mM^{-1} with a mean K_{Sr} for all experiments = 0.27 ± 0.02 mM^{-1} (mean ± s.e.mean, n = 6), a value that is indistinguishable from K_{Sr} calculated on the spare receptor assumption.

Pre-treatment with La^{3+} made protracted experiments such as those shown in Figure 8 somewhat unreliable. Frequently at later phases in the experiment, the log [Ca]-$\bar{m}$ curves appeared to steepen somewhat. To circumvent this effect, two alternative methods were employed in conjunction with eqn. (6). Firstly, complete log [Ca]-$\bar{m}$ curves were not generated after La^{3+} pretreatment but rather dose-ratios at one or two different [Ca] were compared (see Colquhoun (1973) for justification). Secondly, curves such as that shown in Figure 7a were used without La^{3+} pretreatment for levels of [Ca] > 1.8 mM. The results in a later section suggest that this simplification is justified, as e_{Sr}/e_{Ca} is so low as to make eqn. (5a) approach eqn. (6) (see Methods). Both of these alternative approaches were employed in the calculation of the mean K_{Sr} by eqn. (6). It would indeed have been preferable to vary [Sr] and construct Schild plots (Arunlakshana & Schild, 1959) to calculate K_{Sr}. These experiments were impractical technically because they necessitated observing e.p.ps in La^{3+} pretreated, curarized preparations for extended periods. It should be stressed, however, that in the experiments included in the calculation of the mean K_{Sr}, the secondary effects of La^{3+} on $\bar{m}$ were minimized. Furthermore, as the control $\bar{m}_{max}$ could not be restored by increasing [Ca] after La^{3+} treatment in the [La^{3+}] employed (1–2 μM), the calculation of K_{Sr} as an antagonist was not complicated by the presence of spare Ca receptors (see Stephenson & Barlow, 1970; Colquhoun, 1973).

The results in this section demonstrate that the value of K_{Sr} is independent of the method of calculation (if equal $\bar{m}$'s are compared) and suggest, quantitatively that spare Ca binding sites are present on the motor nerve ending.

Determining K_{Ca}

There is only one null method for determining the affinity of full agonists such as Ca (see Colquhoun, 1973); namely, by comparing equal $\bar{m}$ in the presence and absence of an irreversible 'competitive' antagonist (e.g., Waud, 1968). For these experiments control log [Ca]-$\bar{m}$ curves were constructed in the range between 0.25 and 0.8 mM Ca in uncurarized fibres (e.g. Figure 4a filled circles). Preparations were then treated with [La^{3+}]s (1–3.3 μM) that produced an unsurmountable depression of $\bar{m}_{max}$ and the [Ca] increased to generate the new log [Ca]-$\bar{m}$ relationship (Figure 4a filled squares). Now, by eqn. (7a)

$$\frac{1}{[Ca]} = \frac{1}{1-z}\,\frac{1}{[Ca]_{irr}} + \frac{zK_{Ca}}{1-z} \qquad (7a)$$

(where $[Ca]_{irr}$ is the Ca concentration, that after La^{3+} treatment, matches $\bar{m}$ in Ca concentration [Ca] before La^{3+} application and z is the fraction of receptors occluded irreversibly), a plot of $[Ca]^{-1}$ against $[Ca]_{irr}^{-1}$ should be linear (see e.g. Waud, 1968). Figure

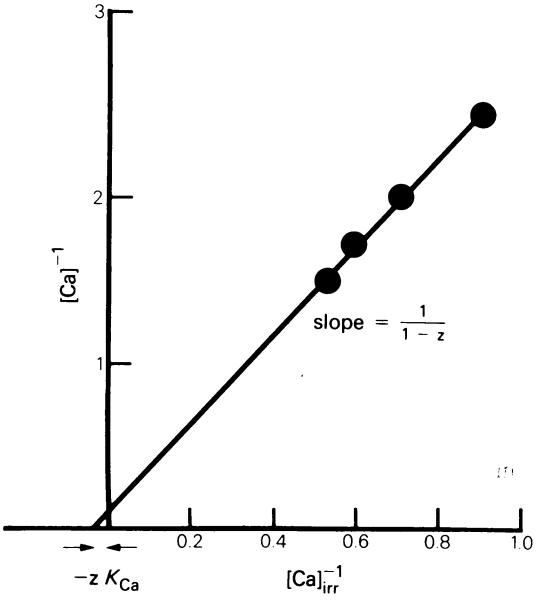

Figure 9 Estimates of the K_{Ca} as an agonist by comparing matching $\bar{m}$ before and after irreversible antognism by La^{3+}. The various parameters of eqn (7a), used to calculate K_{Ca} are shown in the graph. Note, that after determining the fraction of receptors occluded ($z \approx 0.8$) by eqn. (7b), K_{Ca} was estimated from the graph ≈ 0.04 mM^{-1} in this experiment. Direct and indirect estimates suggest K_{Ca} ranges between 0.02 and 0.06 mM^{-1} (see text). Note that this value contrasts to the high K_{Ca} calculated when Ca interacts with another reversible Me agonist (e.g., the high affinity for Ca as an antagonist, see Silinsky, 1977 and Discussion).

9 shows such a plot from the experiment of Figure 4a. Estimating z ≈0.8 from the slope by eqn. (7b), K_{Ca} is roughly 0.04 mM^{-1}. All four experiments of this sort produced similar curves that appeared to intersect the $[Ca]^{-1}$ axis near the origin. Although precise estimates of K_{Ca} are not possible due to the uncertainties inherent in extrapolating reciprocal plots in La^{3+} solutions, the results in the next section suggest that K_{Ca} ranges between 0.02 and 0.06 mM^{-1}.

Estimates of e_{Ca} and e_{Sr}

To determine the efficacy of Ca and Sr, it is first necessary to affix an arbitrary reference point to the stimulus (S), which, as described in the Methods, is equal to product of efficacy and occupancy. If $S = 1$ is defined as the $[Ca]_{50}$ (Stephenson, 1956) then with $S = 1$, $S = e_{Ca}K_{Ca}[Ca]$ (eqn. (3)) reduces to $e_{Ca} = (K_{Ca}[Ca]_{50})^{-1}$ (8b). As $[Ca]_{50}$ and K_{Ca} have been determined, e_{Ca} can be calculated. In the four experiments, e_{Ca} was estimated to be between 9 and 20 (see below).

For Sr, e_{Sr} was determined from the $[Ca]_{50}$ of log [Ca]-$\bar{m}$ curves and the measured intercept in experiments such as shown in Figure 3 using eqn. (9); e_{Sr} = 1/(intercept)($[Ca]_{50}$). In four experiments, e_{Sr} ranged from 0.2–0.5.

In the quantitative aspects of this study, only La^{3+} (and not 2-chloroadenosine) was employed as an irreversible competitive inhibitor of Ca. Although the experimental results suggest that there may be aspects of a competitive relationship between Ca and some reversible adenosine derivatives, a true competitive inhibition is not likely (see e.g., Ginsborg & Hirst, 1972). For example, it has been shown that the maximal level of inhibition by adenosine derivatives (including 2-chloroadenosine) is approximately 50% of the control value (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Silinsky, 1980b) and appears rather independent of the level of ACh release and [Ca]. This multiplicative relationship is suggestive of a non-competitive inhibition and indicates that the irreversible effects of 2-chloroadenosine, in the traditional pharmacological sense, is as an inhibitor of efficacy (Stephenson & Barlow, 1970). Thus, 2-chloroadenosine in addition to demonstrating the presence of spare Ca receptors, can serve as a useful measure of the true value of e_{Ca}. For example, by comparing theoretical curves of varying e_{Ca} at constant K_{Ca} (see Stephenson, 1956, Figure 9; Colquhoun, 1973, Figure 6) and determining the threshold value at which e can be depressed by 50% yet still not decrease $\bar{m}_{max}$, e_{Ca} for Figure 5a can be estimated to be approximately 20. In this experiment and in two other similar experiments with 2-chloroadenosine e_{Ca} ranged from 9 to 20. With this range of e_{Ca} and the $[Ca]_{50}$ values shown in Table 1, eqn. (8a) suggests that K_{Ca} is between 0.02 and 0.06 mM^{-1}.

It thus appears that Ca, although possessing lower affinity as an agonist than Sr, has a 20–100 fold greater efficacy than Sr at presynaptic receptors that mediate depolarization-secretion coupling.

The stimulus (S) *-$\bar{m}$ relationship*

As the results suggest that Ca behaves as a full agonist with high e_{Ca}, it is possible to use eqn. (3) ($S_{Ca} = e_{Ca}K_{Ca}[Ca]$) and the conventional definition of $S = 1$ at $[Ca]_{50}$ (Stephenson, 1956) to generate a complete stimulus-response (S-$\bar{m}$) relationship to describe the events between (occupation) (efficacy) and ACh secretion. With $S = 1$ at $[Ca]_{50}$ (see Figure 10a, dotted lines) then by eqns. (3) and (8a) $S = 2$ at 2.$[Ca]_{50}$, S = 0.5 at 0.5 $[Ca]_{50}$ or generally, $S = X$ at X.$[Ca]_{50}$. The S-$\bar{m}$ relation from the experiment of Figure 1 is shown in Figure 10. Note that the S-$\bar{m}$ relationship is highly complex and non-linear as would be expected from the broad assumption upon which the curve is based, namely that S is linearly related to e and y with non-linearities occurring at subsequent steps in the release

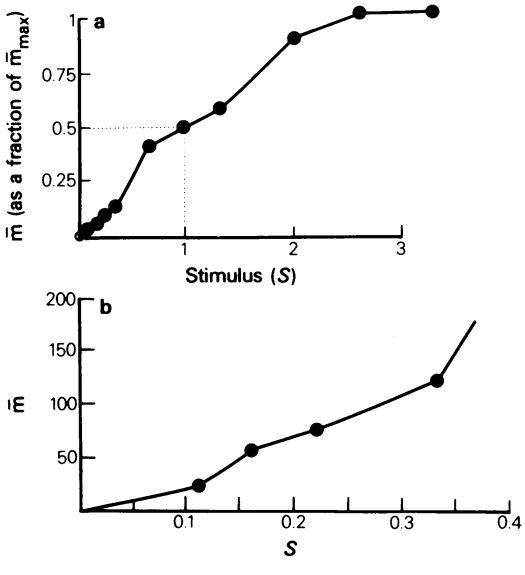

Figure 10 Stimulus-response (S-$\bar{m}$) relationship determined from the experiment shown in Figure 1. If S=1 is arbitrarily defined as = $[Ca]_{50}$ (.....), then by eqn (8a) S=x at x $[Ca]_{50}$. By calculating S at different [Ca], the [Ca]-$\bar{m}$ curve can be transformed into the S-$\bar{m}$ curve shown in the figure. In (a), values of $\bar{m}$ are presented as a fraction of the maximal $\bar{m}$; (b) shows the low range of the S-$\bar{m}$ relation in absolute quanta on an expanded S scale. Although the curve was generated for Ca using the spare receptor assumption, if the theory is correct, it should serve as a link to predict accurately the log [Sr]-$\bar{m}$ curve as well as the log [Ca]-$\bar{m}$ relation.

sequence. The two additional assumptions are that first equal S produces equal $\bar{m}$ and secondly that y, which is normally described by eqn. (1) reduces to K_{Me}[Me] in the case of Ca, (i.e. that the full agonist leaves spare Ca receptors). If these assumptions are justified, then Figure 10 should accurately describe the relation between e_{Me}, y_{Me}, and ACh release, *regardless of the agonist employed.*

Comparison of theoretical and experimental log [Me]-$\bar{m}$ curves

With the framework of modified occupation theory (Stephenson, 1956) and the S-$\bar{m}$ curves of Figure 10 as decoding devices to link $\bar{m}$ with [Me], it should be possible to generate theoretical log [Me]-$\bar{m}$ curves for Ca and Sr that are similar to the curves obtained experimentally. To begin this reconstruction, the values of K_{Sr} = 0.25 mM^{-1}, e_{Sr} = 0.4, K_{Ca} = 0.025 mM^{-1} and e_{Ca} = 15 obtained experimentally were used to calculate S_{Me} at different [Me] as follows:

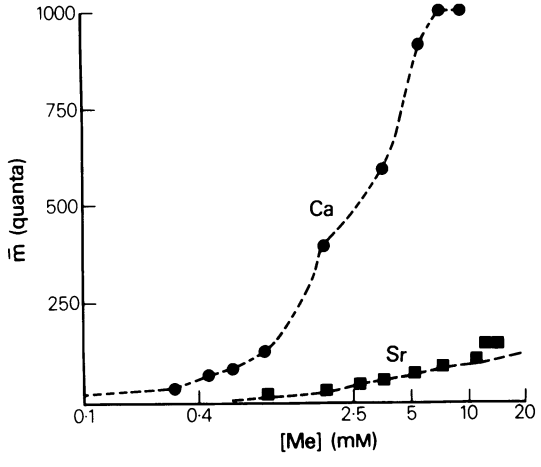

Figure 11 Comparison of theoretical log [Me]-$\bar{m}$ curves (-------) with the experimental results of Figure 1 (● ■). The theoretical curve was determined as follows: firstly, with the experimental values of e_{Sr}=0.4, K_{Sr}=0.25 mM^{-1} (Figure 6), e_{Ca}=15 and K_{Ca}=0.025 mM^{-1} (i.e., e_{Ca} K_{Ca} ≈ 0.37), S_{Ca} was calculated using eqn. (3) and S_{Sr} using S_{Sr}=e·y = e_{Sr} K_{Sr}[Sr]/(1+K_{Sr}[Sr]) at 0.1–0.25 increments in [Me]. The curves of Figure 10 were then employed as links between the calculated S_{Me} and $\bar{m}$. Note that the experimental log [Me]-$\bar{m}$ curves are accurately predicted by modified occupation theory. Similar results were found in the other experiments as well. The shape of the log [Sr]-$\bar{m}$ curve is dependent upon the precise values of e_{Sr}. For example if e_{Sr} = 0.44 (instead of 0.40), then the points near saturation are accurately predicted but at the lower concentrations, the theoretical curve was slightly greater than the actual experimental values. The range of e_{Sr} is dependent upon the precision of $[Ca]_{50}$.

S_{Sr} = $e_{Sr}y_{Sr}$ = $e_{Sr}K_{Sr}$[Sr]/(1 + K_{Sr}[Sr]), and S_{Ca} = $e_{Ca}K_{Ca}$[Ca] (eqn. 3). Next using the calculated S_{Me} values, $\bar{m}$ for that S value was measured from Figure 10. Figure 11 compares the experimental results from Figure 1 (presented here as unconnected experimental points) with the predicted log [Me]-$\bar{m}$ curves (dashed lines) obtained by calculating S at the various [Me]s. It will be observed that the theoretical curves are in excellent agreement with the observed experimental values. Similar results were seen in other experiments as well. Note that the S-$\bar{m}$ curves in Figure 10 were generated from the experiment of Figure 1 for Ca based upon the spare receptor assumption, yet predict accurately the log [Me]-$\bar{m}$ curves for both Sr and Ca (Figure 10). It thus appears that the behaviour of the presynaptic Me binding site, X can be accurately described as that of a pharmacological receptor for depolarization-secretion coupling.

Discussion

General comments

It is evident from these results that despite the elegant simplicity of the original quantitative studies on the hypothetical Ca receptor (X) (Dodge & Rahamimoff, 1967; Meiri & Rahamimoff, 1971), the assumption that the ACh secretion obeys the law of mass action with respect to the occupancy of X is not in accord with the present results. Firstly, the surmountable relationship between Ca and irreversible antagonists suggest the presence of spare Ca binding sites. Secondly, the affinity constant for the partial agonist Sr appears to have essentially the same value when calculated by several methods, including those that do and those that do not require the spare receptor assumption for their application. Finally, there appears to be excellent agreement between the log[Me]-$\bar{m}$ curves obtained experimentally and those reconstructed in accordance with modified occupation theory (Figure 11), the theory which forms the basis for the original concept of spare receptors (Stephenson, 1956).

To illustrate the inadequacy of the mass action formulation for Ca numerically, say 50% of the total [X] are occupied by Ca (y = 0.5). If a fourth power relationship held for the entire [Ca]-$\bar{m}$ relation, then $\bar{m}$ should be only $(0.5)^4$ or 6% of $\bar{m}_{max}$. In contrast, the results of this study suggest precisely the opposite conclusion, namely that for K_{Ca} = 0.04 mM^{-1} (Figure 10) and $[Ca]_{50}$ = 2.6 mM (Table 1), 50% of $\bar{m}_{max}$ would be achieved with only 9–10% of the X sites occupied, in turn leaving 90–91% of the receptors spare. In addition, it appears evident from the complexity of the log [Me]-$\bar{m}$ curves presented here (see also below)

and from observations by others at normal physiological levels of [Ca] and ACh release (Jenkinson, 1957; McLachlan, 1978), that the major part of the log [Me]-$\overline{m}$ curve is not accurately described by a fourth power relation. As a final comment about the mass action assumption, it should be stressed that affinity constants for competitive antagonists (e.g Mg, Co and even Sr, in Figure 8) calculated by mass action principles using some form of the Schild equation (e.g. Jenkinson, 1957; Dodge & Rahamimoff 1967; Crawford, 1974) can serve as effective means of identifying the site of action of a particular activator of ACh secretion (for review, see Silinsky & Mellow, 1981).

It may be argued that in contrast to selective binding non-specific screening effects of Me on fixed surface charges near the external surface of ion channels may depress excitability and thus contribute to the experimentally-determined K_{Me} values. For Na^+ channels, this is unlikely as n.t.p. amplitudes are essentially indistinguishable in a variety of [Me]s $\geq m_{max}$. Furthermore, when experiments such as those shown in Figure 7a are extended to low [Ca], it was found that the two curves intersected at [Ca] between 0.4 and 0.9 mM ($n = 4$). If Sr acts as a partial agonist on the same binding site as Ca and produces no substantial secondary effects (such as screening), then theoretically the two curves should intersect at a value near the $\overline{m}_{max}$ for the log [Sr]-$\overline{m}$ curve (see Colquhoun, 1973). This prediction has been borne out experimentally; in all 3 experiments (Table 1, experiments 1–3, A–C) $\overline{m}_{max}$ for Sr approximated $\overline{m}$ observed in 0.9 mM Ca (e.g. Figure 1). It thus appears that non-selective screening effects on Na^+ channels do not play a significant part in the quantitative results. It is possible, however, that the failure to restore $\overline{m}_{max}$ for Ca in two of three experiments with 2-chloradenosine may be due to an effective closure of some Na channels at the very high (15–20 mM) Ca concentrations employed (cf. Meiri & Rahamimoff, 1971). With respect to Ca channels, it does not appear that screening of Ca channels by Me contribute significantly to determinations of K_{Me} and e. The depressant effect of high Me on Ca channels as predicted by the screening effect was not observed when the membrane was depolarized by electrical stimulation (Cooke & Quastel, 1973). The inhibitory effects that have been interpreted as screening, were only seen with potassium-depolarized preparations. These effects, rather than representing a generalized influence of high Me on depolarization-sensitive Ca channels, more probably reflect a specific interaction between K^+ and Ca (Cooke & Quastel, 1973). It appears then, that the binding of Me agonists to the motor nerve ending Ca receptor remains the simplest single interpretation of depolarization-secretion coupling (see Silinsky, 1977; Silinsky & Mellow, 1981).

K_{Ca} as an agonist (0.02–0.06 mM^{-1}) is approximately one-hundredth the K_{Ca} reported as an antagonist of asynchronous, Ba-mediated evoked ACh release (Silinsky, 1977). It is possible that this high affinity antagonism by Ca of asynchronous release occurs at a different locus from that responsible for Ca-mediated $\overline{m}$. Alternatively, the high Ca as an antagonist might reflect the true affinity for the closed Ca channel (or X site) whilst K_{Ca} as a full agonist may represent an apparent, Briggs-Haldane affinity constant (Segel, 1975) for the open Ca channel. It is of interest that if (i) the Ca channel or X site exists in two states, a closed, inactive state that does not support synchronous release and an open, active state that is associated with $\overline{m}$, and (ii) the ratio of inactive to active X is high in the absence of Ca (when the membrane is depolarized), then both the low K_{Ca} as an agonist and the high K_{Ca} as an antagonist can be predicted by a linear, two-state model to describe S (unpublished). In such a scheme, the value of K_{Sr} would be independent of whether the partial agonist Sr was employed as an agonist or antagonist (Colquhoun, 1973). Further details will be provided in a subsequent communication.

When Hill plots (Brown & Hill, 1923) of log [Me]-$\overline{m}$ curves were constructed, they had slopes of 2–3 for much of the curves and also exhibited regions of negative cooperativity, which for Ca generally occurs around the range of the normal [Ca]. This negative cooperativity is described mathematically by a model in which membrane subunits interact in a sequential fashion (Koshland, Nemethy & Filmer, 1966). It seems possible, however, that the flattening of the log [Ca]-$\overline{m}$ curve in the region of normal Ca may reflect the saturation of one Ca-dependent process and the emergence of another, lower potency Ca-dependent process, both processes being blocked competitively by Co, Mg, and Mn (Jenkinson, 1957; Dodge & Rahamimoff, 1967; Meiri & Rahamimoff, 1972; Weakley 1973; Crawford, 1974). Because of this uncertainty, all comparisons of equal $\overline{m}$ were made at [Ca]<1 mM. Indeed, deviations in the straight line predicted by eqn. (4) begin at ~ 1 mM Ca. This deviation may reflect the possibility that the spare receptor assumption is inapplicable at Ca≥ 1 mM or that another Ca-dependent process begins to emerge. Further experiments are in progress to determine the nature of this apparent negative cooperativity.

Physical correlates of affinity and efficacy

In view of these and previous experimental results (e.g., Meiri & Rahamimoff, 1971), it appears simplest to suggest that K_{Me} represents the affinity of Me for the site of alkaline earth cation entry, namely the Ca channel. For example, the K_{Sr} as an antagonist under conditions where Sr does not support ACh release is for a common site shared by Ca and Sr that

precedes the release site, presumably the Ca channel. Indeed, Sr- and Ca-mediated ACh release are both initiated through the same Ca channel (for review, see Silinsky & Mellow, 1981). Furthermore, affinity constants for Mg, Co and Mn ions are for the site of Me entry as these antagonists when applied directly to the motor nerve ending cytoplasm (using liposomes) do not depress $\bar{m}$ (Kharasch *et al.*, 1981). It is thus likely that the action of Sr as a competitive inhibitor is also at the site of Ca entry. As K_{Sr} as an agonist is indistinguishable from the K_{Sr} as an antagonist of the same release process, it appears reasonable to suggest that all K_{Me} values reflect behaviour at the Ca channel, apart from any activity at intraterminal release sites. It is of interest that, Ca entry in neurones has been shown to be linearly related to y_{Ca} (Brown, Akaike & Lee, 1978; Nachsen & Blaustein, 1979). Accordingly, if y (and thus K_{Me}) is for the Ca channel, then as the [Me] is varied, $S (= e.y)$ would be linearly related to y and provide justification for one of the major assumptions of this study, namely that non-linearities in the log [Me]-$\bar{m}$ curves are introduced beyond the site of receptor (or channel) occupation and thus beyond S.

For e_{Me}, one tempting suggestion is that the sequence $e_{Ca} > e_{Sr} > e_{Ba} > e_{Mg}$ represents the relative mobility of the ion in the Ca channel (Meiri & Rahamimoff, 1971; Silinsky, 1978). Although not excluding this interpretation it should be stressed that studies on Ca currents do not agree with this mobility sequence (e.g. Hagiwara, Fukuda & Eaton, 1974). Moreover, the same relative selectivities of Ca, Sr and Mg for *synchronous* ACh release are observed using ion-containing liposomes to bypass the Ca channel (Silinksy, 1981). It seems reasonable to suggest, therefore, that e_{Me} reflects the potency (possibly affinity) of the Me species at intraterminal locus, the release site. This further reinforces the distinction made previously (see e.g. Katz, 1969; Crawford 1974; Martin, 1977; Silinsky, 1977) between an extracellular site involved in regulating divalent cation entry (the X site) and the intraterminal release site(s) assuming that Ca enters the nerve ending through ion channels.

Conclusions

The following is a plausible scheme for the process of depolarization-secretion coupling consistent with the findings presented here. For the full agonist, Ca, although the K_{Ca} for the agonist state is very low (0.02–0.06 mM) and the apparent occupancy small when the Ca channel is opened, this small occupancy can produce very large levels of ACh release due to the high e_{Ca} at an intraterminal site. If e reflects the affinity of Me for an intraterminal membrane release site, then despite the small open channel occupancy, a large concentration of bound Ca will be strategically located near release sites and produce large quantities of ACh secretion as compared to Sr at the same concentration. At the motor nerve ending as in other receptor-effector systems, (Ariëns, 1979) the maximal response of the system may be limited at the output end by the number of available ACh quanta or activatable attachment sites. By this scheme, spare receptors are equivalent to spare Ca channels, i.e. many more Ca channels are present than necessary to produce $\bar{m}_{max}$ because the limiting step (quantal ACh discharge) occurs beyond the initial site of Ca binding. Speculatively, the non-linearity of the S-$\bar{m}$ curve could be due to a complex relationship between intracellular Ca and a calmodulin-like protein associated with e.g. synaptic vesicles. (For an application of these results to other secretory systems, see Silinsky, 1981).

It is noteworthy that if e_{Ca} reflects an intraterminal affinity constant, then the selectivity of Me agonists for *neurally*-evoked asynchronous but not liposome or high K^+-evoked ACh release can be explained simply within the same general framework applied in this study to the synchronous release process (Silinsky & Mellow, 1981). Possibly, the effect of adenosine derivatives in decreasing efficacy could reflect a decrease in the affinity of intraterminal release sites (or synaptic vesicles or both) for Ca, modulated through externally-situated adenosine receptors on adenylate cyclase (Silinsky, 1980b). With the pharmacological approach described in this study, it seems possible to circumvent the problems inherent in binomial statistical approaches to ACh release (McLachlan, 1978) yet provide a quantitative handle on the complex process of depolarization-secretion coupling.

To conclude, it appears that the mathematical framework of receptor theory provides a unifying explanation for the behaviour of Ca and Sr consistent with the experimental log [Me]-$\bar{m}$ curves and with the evidence that spare Ca receptors exist at cholinergic motor nerve ending. Further experiments are necessary to determine which particular theory of the receptor provides the most accurate description of the effects of alkaline earth cations on both synchronous and asynchronous forms of neurotransmitter secretion.

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