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AN ESTIMATE OF THE EQUILIBRIUM DISSOCIATION CONSTANT FOR CALCIUM AS AN *ANTAGONIST* OF EVOKED ACETYLCHOLINE RELEASE: IMPLICATIONS FOR EXCITATION-SECRETION COUPLING

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The equilibrium dissociation constant (K_d) for Ca^{2+} as an antagonist of evoked acetylcholine (ACh) release was determined in the hope of distinguishing whether divalent cations control excitation-secretion coupling selectively (by *binding* with high affinity to an external membrane site) or non-selectively (by *screening* fixed negative charges on the external surface of the nerve terminal). ACh release was detected electrophysiologically by means of conventional intracellular recording techniques at frog motor endplates. Ba^{2+} was used as the agonist to support the asynchronous release of ACh by repetitive motor nerve impulses. Despite its dispersed nature, release mediated by Ba^{2+} occurs through the same conductance pathway as synchronous release mediated by Ca^{2+}. Ca^{2+} was found to be a potent antagonist of Ba^{2+}-dependent release with a $K_d = 0.12 \pm 0.02$ mM (mean ± s.e. mean, $n = 5$). This value is 30–50 times lower than the K_d for Mg^{2+} as an antagonist of the same release process. It is suggested that antagonism of release by Ca^{2+} is likely to be exerted at the same external site that binds other divalent cation antagonists, a site that appears essential for the agonist behaviour of Ca^{2+}. The high affinity (low K_d) of Ca^{2+} as an antagonist of ACh release suggests that a selective, binding model appears to be the most appropriate single description of the action of divalent cations at the external surface of the motor nerve ending.

Introduction Studies on the behaviour of divalent cations as mediators and inhibitors of excitation-secretion coupling have generally relied upon the mathematical framework of traditional drug receptor theory (Stephenson & Barlow, 1970). For example, the effect of Ca^{2+} as an agonist for the release of acetylcholine (ACh) by motor nerve impulses (Katz, 1969) is inhibited competitively by antagonists such as Mg^{2+} (Castillo & Engbaek, 1954; Castillo & Katz, 1954; Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968; Crawford, 1974), Co^{2+} (Weakly, 1973; Crawford, 1974) and Mn^{2+} (Meiri & Rahamimoff, 1972) and by the partial agonist, Sr^{2+} (Meiri & Rahamimoff, 1971). Furthermore, equilibrium dissociation constants (K_ds) for these antagonists, when determined by comparing *equal* responses (Gaddum, 1957) have provided a powerful means of identifying the external receptive site that regulates evoked ACh release. However, with respect to the agonist the only means of estimating the K_d for Ca^{2+} has been by extrapolation of reciprocal dose-response curves (Lineweaver & Burk, 1934) a method wrought with untestable assumptions at the neuromuscular junction (Stephenson & Barlow, 1970). In addition, while it is likely that the releasing site activated by Ca^{2+} is near the internal surface of the nerve terminal, the antagonism of release by other divalent cations, appears to be exerted at an external site (see Crawford, 1974).

Because of these complexities, it has been suggested that an ion such as Mg^{2+}, rather than selectively *binding* to a negatively charged site in the fashion of a drug-receptor interaction, may inhibit release by non-selectively *screening* fixed negative charges associated with the external surface of the nerve terminal, thereby reducing the surface potential and the surface $[Ca^{2+}]$ near the Ca^{2+} conductance pathway (Muller & Finkelstein, 1974). This interpretation implies that Ca^{2+}, in asserting its role as the physiological mediator of excitation-secretion coupling, need not bind to an external site on the motor nerve ending.

As the true affinity of the external binding site for Ca^{2+} is unknown, there is at present no means of distinguishing between the binding and screening models. It would thus be of interest to have a method for determining the K_d for Ca^{2+} as an antagonist of evoked ACh release. If this value were considerably lower than the K_d for Mg^{2+}, and represented antagonism at the same site as Mg^{2+}, then the screening model (which predicts that ions of equal valency are equipotent in their effects) would be rendered untenable (Muller & Finkelstein, 1974).

Methods The isolated nerve-cutaneous pectoris preparation of the frog was studied by the use of conventional electrophysiological methods for nerve stimulation and intracellular microelectrode recording (for precise details, see Silinsky, 1977a, b). Preparations were first washed with normal Ringer solution (mM: NaCl 115, KCl 2, $NaHCO_3$ 2, $CaCl_2$ 1.8) and then equilibrated for 2 h in flowing Ringer

solution in which 0.25 mM $BaCl_2$ was substituted for $CaCl_2$ in normal Ringer solution (Ba^{2+} (0.25 mM) Ringer solution). Ba^{2+} (0.25 mM) Ringer was used as the control solution, the Ba^{2+} ion serving as the agonist for evoked ACh release. With the exception of normal Ringer solutions, all solutions contained neostigmine methyl sulphate (1 μg/ml) to increase the amplitude of the miniature end-plate potentials (m.e.p.ps). Other solutions (see text) had approximately the same univalent ion concentration as normal Ringer solution but varying concentrations of divalent cations.

Results Ba^{2+} has been shown recently to be an effective agonist for the asynchronous release of ACh by repetitive nerve impulses (Silinsky, 1977a). Despite the differences in temporal behaviour between release mediated by Ba^{2+} and by Ca^{2+}, K_d measurements for the antagonists Co^{2+} and Mg^{2+} suggest that Ba^{2+} couples excitation to secretion through the same conductance pathway as Ca^{2+} (Silinsky, 1977b). Ba^{2+} thus appears to be an appropriate choice of agonist in this study.

The ability of Ba^{2+} to support evoked ACh release is shown in Figure 1. In Figure 1a, brief repetitive nerve stimulation (25 Hz) in Ba^{2+} (0.25 mM) Ringer solution caused m.e.p.ps to occur at such a high frequency (⩾100/s) that they produced a slow depolarization of the postsynaptic membrane. This slow depolarization is proportional to the number of ACh quanta released *asynchronously* and can be used to determine the frequency of m.e.p.ps (see Heuser & Miledi, 1971; Katz & Miledi, 1972; Silinsky, 1977b). However, for the present purposes it is only necessary to compare *equal* slow depolarizations in the presence and absence of antagonist to determine the K_d for the antagonist.

Compared to Ba^{2+}, Ca^{2+} is a weak partial agonist in the process of asynchronous evoked release (Silinsky, 1977b). As both Ca^{2+} and Ba^{2+} support asynchronous release through the same conductance channel (Miledi & Thies, 1971; Silinsky, Mellow & Phillips, 1977), Ca^{2+} should be an antagonist (Stephenson, 1956) of Ba^{2+}-mediated release. Figure 1b shows this to be the case. In this trace, although the addition of 0.25 mM Ca^{2+} to Ba^{2+} (0.25 mM) Ringer solution produced endplate potentials (e.p.ps), this concentration of Ca^{2+} almost fully antagonized the slow depolarization associated with asynchronous, Ba^{2+}-mediated release. The Ba^{2+}-Ca^{2+} relationship appears competitive; increasing the $[Ba^{2+}]$ to 0.75 mM (Figure 1c) caused the underlying envelope of the e.p.ps to depolarize slowly over a similar time course and to the same absolute magnitude as the trace in Figure 1a. The K_d of Ca^{2+} as a competitive antagonist can now be calculated from the following equation:

$$K_d = [Ca^{2+}]/[(Ba_A^{2+}/Ba_0^{2+}) - 1]^{-1} \quad \text{(Gaddum, 1957)}$$

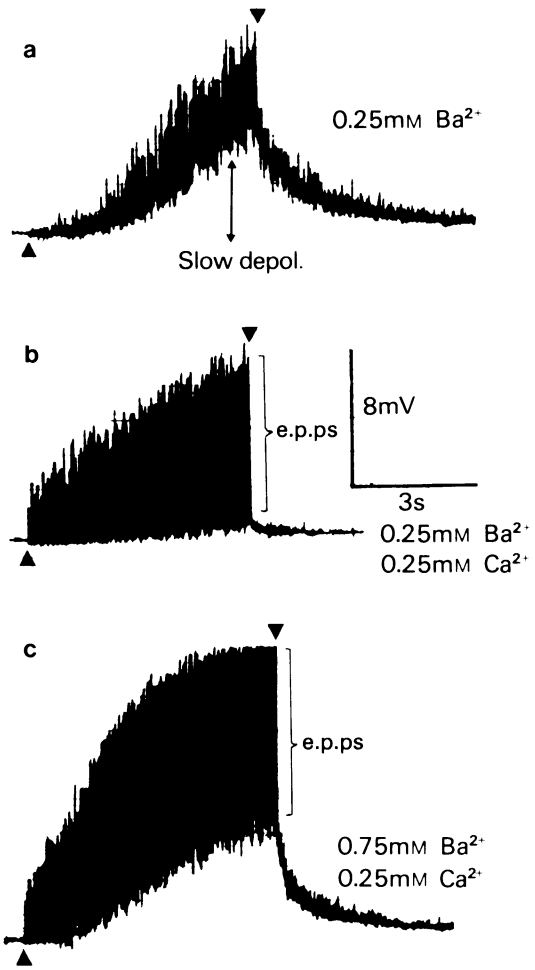

Figure 1 Antagonism of evoked acetylcholine release by Ca^{2+}. Stimuli delivered at 25 Hz for 5–6 seconds. Triangles indicate start and finish of stimulation. (a) 0.25 mM Ba^{2+} Ringer-stimulation produced a large increase in miniature end-plate potential frequency and an associated slow depolarization (slow depol.) of the post-synaptic membrane. This represented the control response with Ba^{2+} serving as the agonist. (b) 0.25 mM Ba^{2+} + 0.25 mM Ca^{2+} Ringer-addition of Ca^{2+}, while now producing end-plate potentials (e.p.ps), eliminated almost completely the slow depolarization associated with Ba^{2+}-mediated asynchronous release. (c) 0.75 mM Ba^{2+} + 0.25 mM Ca^{2+} Ringer. Elevation of the $[Ba^{2+}]$ surmounted the antagonism of the slow depolarization by Ca^{2+}. Note that the underlying envelope of the e.p.ps follows a similar time course and reaches the same absolute amplitude as the control trace in (a). Duration of stimulation period was extended slightly in (c) to illustrate that the slow depolarization has approached a steady-state value. Resting potential for all traces was −40 mV. Records were made by Brush-Gould (Model 220) pen recorder. For full description of experimental procedures, see Silinsky, 1977a, b.

where Ba_0^{2+} is the $[Ba^{2+}]$ that produced the control response and Ba_A^{2+} is the $[Ba^{2+}]$ that produced a matching response in the presence of the antagonist, Ca^{2+}.

Thus, from the experiment of Figure 1,

$$K_d = [0.25]/[(0.75/0.25) - 1]^{-1} \text{ or } 0.125 \text{ mM}$$

The mean K_d in five experiments was 0.12 ± 0.02 mM (mean $\pm$ s.e. mean).

Discussion The K_d for Ca^{2+} as an antagonist of evoked ACh release (0.12 mM) is an order of magnitude lower than the values obtained by extrapolation of reciprocal dose-response curves (Dodge & Rahamimoff, 1967; Crawford, 1974) and 30–50 times lower than the K_d for Mg^{2+} as an antagonist of the *same* release process (Dodge & Rahamimoff, 1967; Crawford, 1974; Silinsky, 1977b). As this value was obtained by comparison of equal responses in the presence and absence of the antagonist, it presumably represents a more reliable estimate of the K_d than those values obtained by other methods (for assumptions see Gaddum, 1957; Stephenson & Barlow, 1970). It thus appears that Ca^{2+} binds with high affinity (low K_d) to a site involved in excitation-secretion coupling. As recent results suggest that divalent cations, *once through the nerve terminal membrane,* may be of nearly equal effectiveness in supporting asynchronous release (Kita & Van der Kloot, 1976), it seems unlikely that an intraterminal site is the locus of this high affinity antagonism by extracellular Ca^{2+}. Furthermore, as Mg^{2+}, Co^{2+} and Mn^{2+} antagonize excitation-secretion coupling by acting externally on a site that appears essential for initiating the agonist behaviour of Ca^{2+}, it is simplest to suggest that Ca^{2+} also binds to this same external site.

In conclusion, while not eliminating the possibility that a small component of screening contributes to the observed activity, these results do suggest that the binding model is still the most appropriate single description of the regulation of excitation-secretion coupling at the external surface of the motor nerve terminal.

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