Br. J. Pharmac. (1978), **63**, 229–232

ON THE CONDUCTANCE PATHWAY TRAVERSED BY STRONTIUM IN MEDIATING THE ASYNCHRONOUS RELEASE OF ACETYLCHOLINE BY MOTOR NERVE IMPULSES

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A study was made to determine whether the Sr^{2+}-dependent *asynchronous* release of acetylcholine by nerve impulses is mediated by the conventional Ca^{2+} conductance channel or, as has been suggested recently, through an alternative ion pathway. Experiments were performed on the frog neuromuscular junction by the use of standard electrophysiological techniques. Repetitive nerve stimulation in Sr^{2+}-Ringer solutions caused a marked increase in miniature end-plate potential (m.e.p.p.) frequency which was dependent on Sr^{2+} concentration and inhibited in a competitive fashion by the known Ca^{2+} antagonists, Co^{2+} and Mg^{2+}. The equilibrium dissociation constants (K_ds) determined for both Co^{2+} (0.09 ± 0.01 mM, mean ± s.e. mean, $n = 5$) and Mg^{2+} (3.7 ± 0.3 mM, mean ± s.e. mean, $n = 4$) were essentially the same as the reported values for these antagonists in blocking Ca^{2+}-mediated transmitter release by nerve impulses. These results suggest that Sr^{2+} mediates asynchronous evoked transmitter release through the conventional calcium conductance channel.

Introduction The movement of Ca^{2+} from the extracellular fluid through the presynaptic nerve membrane acts to couple depolarization to secretion at motor nerve terminals (Katz, 1969). One electrophysiological expression of such evoked, Ca^{2+}-dependent neurosecretion is the endplate potential (e.p.p.), which is thought to reflect the synchronous release of many acetylcholine (ACh) quanta from discrete releasing sites along the nerve ending. A second manifestation of evoked ACh release is the delayed increase in miniature endplate potential (m.e.p.p.) frequency that occurs upon repetitive nerve stimulation (del Castillo & Katz, 1954; Braun, Schmidt & Zimmermann, 1966). This asynchronous discharge of single ACh quanta is also dependent on extracellular Ca^{2+} (Miledi & Thies, 1971; Hurlbut, Longnecker & Mauro, 1971) in a concentration-dependent fashion (Silinsky, Mellow & Phillips, 1977).

It has been shown (Miledi, 1966; Dodge, Miledi & Rahamimoff, 1969; Meiri & Rahamimoff, 1971) that Sr^{2+} can substitute for Ca^{2+}, although far less effectively, in supporting the process of synchronous transmitter release. With respect to asynchronous release, a recent study on preganglionic nerve terminals (McLachlan, 1977) demonstrated enhancement of Ca^{2+}-mediated asynchronous release by strontium. The author suggests that in coupling depolarization to asynchronous transmitter secretion, Sr^{2+} (and other divalent cations) enters the nerve terminal via an alternative pathway to the Ca^{2+} conductance channel mediating synchronous release.

As Ca^{2+} (Silinsky *et al.* 1977) and Ba^{2+} (Silinsky, 1978) both mediate asynchronous release through the conventional Ca^{2+} channel, it seemed curious that Sr^{2+} would act through a separate pathway. It was therefore decided to investigate the effects of the conventional Ca^{2+} antagonists Co^{2+} and Mg^{2+} (Baker, 1972) on Sr^{2+}-dependent asynchronous release at the frog neuromuscular junction.

Methods All experiments were performed on the isolated nerve-cutaneous pectoris preparation of the frog, by means of conventional electrophysiological methods for nerve stimulation and intracellular microelectrode recording (for details, see Silinsky, 1977; 1978; Silinsky *et al.* 1977). Preparations were washed initially with a standard Ringer solution of the following composition (mM): NaCl 115, KCl 2, $NaHCO_3$ 8, $CaCl_2$ 1.8 (pH 6.8 to 7.2). Sr^{2+}-Ringer was identical to the standard Ringer except that $SrCl_2$ was substituted for $CaCl_2$. The antagonists Co^{2+} and Mg^{2+} were added as the chloride salts. In the experiments with $MgCl_2$, changes in resting m.e.p.p. frequency (due presumably to osmotic effects of high concentrations of the salt) were minimized by the addition of appropriate amounts of sucrose to the control solutions. All Sr^{2+} solutions contained neostigmine methylsulphate (1 μg/ml) to increase the amplitude of the m.e.p.ps. Preparations were equilibrated with flowing Sr^{2+}-Ringer solution for 15 to 30 min before the start of an experiment. Trains of stimuli were delivered to the nerve for 10 s at rates varying from 2.5 to 15 hertz M.e.p.p. frequency during a train was measured for the last 8 s of stimulation (steady-state levels of m.e.p.p. discharge appeared to be reached within 1 to 2 s of stimulation). Responses phase-locked to the stimulus (i.e. e.p.ps) were not included in the measurements.

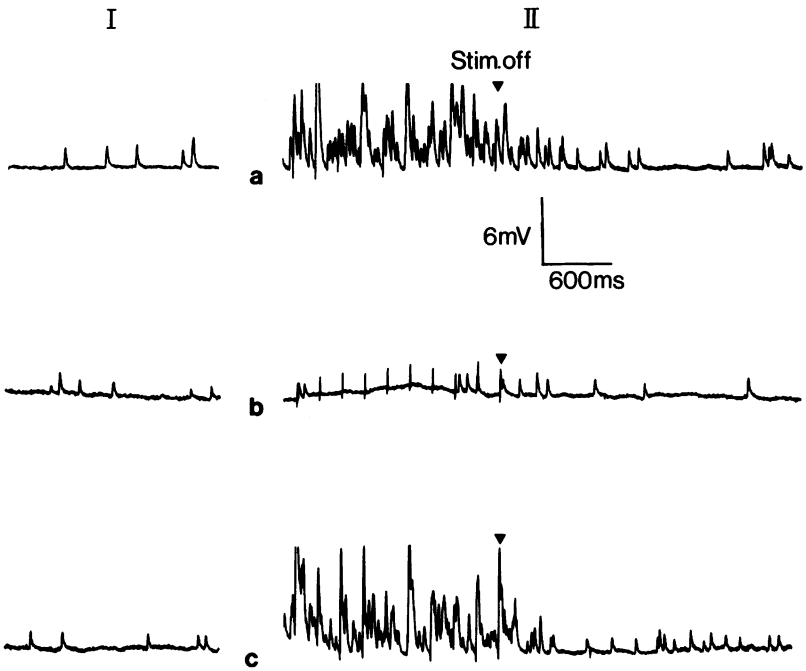

Figure 1 Sr^{2+}-mediated asynchronous transmitter release and its antagonism by Co^{2+}. All records are photographs of pen recorder traces. The traces in column I represent the resting condition; resting m.e.p.p. frequency was 2/s for (a) (b) and (c). Traces in column II show the last 2 s of 10 s trains of stimuli delivered to the nerve at 5 hertz. Each trace in column II has, in addition, records of the 2.5 s immediately following the end of stimulation, which is denoted by an arrow. Evoked m.e.p.p. frequencies were measured as described in the text. (a) In 1.0 mM Sr^{2+}-Ringer solution. Stimulation produces an increase in m.e.p.p. frequency to a level of 13.8/second. (b) In 1.0 mM Sr^{2+} + 0.2 mM Co^{2+} Ringer. Note that the response is virtually abolished, with an evoked m.e.p.p. frequency of 2.8/second. (Spikes occurring during early part of train at 200 ms intervals represent stimulus artifacts). (c) In 3.5 mM Sr^{2+} + 0.2 mM Co^{2+} Ringer. The antagonism by Co^{2+} is surmounted (above the control value) by an increased Sr^{2+} concentration, the evoked m.e.p.p. frequency being 16.0/second. (Note that Co^{2+} also antagonizes e.p.ps and this antagonism is also surmounted by an increased Sr^{2+} concentration.) Similar records were obtained when Mg^{2+} was used as the antagonist.

Results Figure 1 illustrates a representative experimental result. In Figure 1a, stimulation in 1.0 mM Sr^{2+}-Ringer solution, in addition to producing e.p.ps of low quantal content (see Dodge *et al.*, 1969), evoked a marked increase in m.e.p.p. frequency, from a resting level of 2/s (I) to 13.8/s (II) during stimulation. Addition of the Ca^{2+} antagonist Co^{2+} (0.2 mM) depressed both the e.p.ps and the evoked m.e.p.p. discharge (Figure 1b). Increasing the Sr^{2+} concentration to 3.5 mM surmounted the antagonism by Co^{2+} (Figure 1c), suggesting that Co^{2+} is acting as a competitive antagonist of Sr^{2+} in the process of asynchronous transmitter release. Indeed, in several experiments it was possible to construct a log dose-response curve for Sr^{2+}-evoked m.e.p.ps and to observe a parallel shift in this curve in the presence of Co^{2+}, adding support to this contention. The equilibrium dissociation constant (K_d) for Co^{2+} as a competitive antagonist may be calculated from the following equation (Gaddum, 1957):

$$K_d = [Co^{2+}]([Sr_2^{2+}]/[Sr_1^{2+}] - 1)^{-1}$$

where $[Sr_1^{2+}]$ is the Sr^{2+} concentration that produces a given evoked m.e.p.p. frequency and $[Sr_2^{2+}]$ is the Sr^{2+} concentration producing the same evoked m.e.p.p. frequency in the presence of a concentration, $[Co^{2+}]$, of the antagonist. In five separate experiments, the mean K_d value for Co^{2+} was found to be 0.09 ± 0.01 mM (mean ± s.e. mean). This value is similar to the reported values for Co^{2+} as an antagonist of calcium-dependent synchronous (0.18 mM, Weakly, 1973; 0.07 mM, Crawford, 1974) and asynchronous ACh release (0.13 mM, Silinsky *et al.*, 1977).

In a series of similar experiments, Mg^{2+} competitively antagonized the evoked discharge of m.e.p.ps in Sr^{2+}-Ringer with a mean K_d value, calculated by the above equation, of 3.7 ± 0.3 mM (mean $\pm$ s.e. mean, $n = 4$). This value compares favourably with reported values for Mg^{2+} as an antagonist of synchronous (4.0 mM, Jenkinson, 1957; 3.0 mM, Dodge & Rahamimoff, 1967; 4.4 mM, Crawford, 1974) and asynchronous (4.6 mM, Silinsky *et al.*, 1977) Ca^{2+}-dependent ACh release.

Discussion Our results indicate that strontium, in a concentration-dependent fashion, can effectively support the asynchronous evoked discharge of transmitter quanta. This is in agreement with the results of Dodge *et al.* (1969) who demonstrated a delayed increase in m.e.p.p. frequency in the first 100 ms following a single stimulus. The determination of K_d values for antagonists of alkaline earth cation entry into the nerve terminal provides a means of identifying pharmacologically the site of ion entry. We have shown that Sr^{2+}-mediated evoked asynchronous release is inhibited in a competitive fashion by the traditional antagonists of Ca^{2+} entry, Mg^{2+} and Co^{2+}. Furthermore, the K_d values for Co^{2+} and Mg^{2+} as antagonists of Sr^{2+}-dependent release are essentially the same as the respective values for the antagonistic effects of these ions on Ca^{2+}-mediated release. These data suggest, then, that strontium is acting to support asynchronous release through the same conductance pathway as that involved in calcium-dependent synchronous (Katz, 1969) and asynchronous (Silinsky *et al.*, 1977) neurosecretion. It has recently been shown that barium, which cannot support synchronous release (Silinsky, 1977), is a powerful agonist for asynchronous release and acts through the conventional calcium conductance channel (Silinsky, 1978). All of these results, then, appear at variance with the conclusions drawn from experiments on ganglia by McLachlan (1977), who visualizes two separate release mechanisms (synchronous or 'phasic' and asynchronous or 'residual'), each coupled to cation influx through a different channel in the nerve terminal membrane. It is probable that at least two separate evoked transmitter release mechanisms (with differential divalent cation sensitivities) do exist at cholinergic nerve terminals (as suggested by McLachlan, 1977). However, with respect to the motor nerve ending the separation does not appear to be at the level of the conductance pathway in the presynaptic membrane, but may involve different intraterminal ionic binding (or screening) sites (Silinsky & Mellow, 1978).

This work was supported by U.S. Public Health Service Grant NS 12782 and Training Grant GM 07263.

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(*Received March 7, 1978*)