Cobalt Blocks the Decrease in MEPSP Frequency on Depolarization in Calcium-free Hypertonic Media

LUCA LANDÒ*, JOSEPH GIOVANNINI, and ROBERT S. ZUCKER

Department of Physiology-Anatomy, University of California, Berkeley, California 94720

SUMMARY

Media made hyperosmotic with sucrose increase the frequency of spontaneously released quanta of transmitter, or miniature excitatory postsynaptic potentials (MEPSPs). In calcium-free medium, depolarization with high potassium reduces the MEPSP frequency, presumably due to calcium efflux in the reversed gradient condition. This effect of depolarization is blocked by cobalt, supporting the above interpretation of the effects of hypertonicity and depolarization and suggesting that cobalt can block efflux as well as influx through calcium channels.

INTRODUCTION

Spontaneous release of neurotransmitter is strongly affected by the osmotic pressure of the external solution (Fatt and Katz, 1952; Furshpan, 1956; Blioch et al., 1968). This phenomenon, sometimes called hyperosmotic neurosecretion, can be a useful method to study the mechanism of transmitter release. One example is the ability to release neurotransmitter in the absence of nerve terminal depolarization. The processes underlying this event are presently unknown, although many theories have been proposed (Bass and Moore, 1966; Blioch et al., 1968; Hubbard et al., 1968; Kita and van der Kloot, 1977; van der Kloot and Kita, 1973; Alnaes and Rahamimoff, 1975).

We have further investigated this problem and found that an elevation of internal calcium is the most adequate explanation. We add experimental evidence to this interpretation and debate an argument raised against it.

We found that cobalt, a cation known to block the inflow of calcium through voltage-controlled membrane channels, may also be effective in blocking the outflow of calcium from the nerve terminal.

METHODS

Experiments were performed on the abductor of the dactylopodite (corresponding to the claw opener muscle) of the first and second walking leg of the crayfish *Procambarus clarkii*. The leg was removed close to the autotomy point and fixed on the Sylgard bottom of a small Petri dish. The dorsal surface of the exoskeleton was separated from the propodite, and the adductor (closer) muscle was carefully removed.

* To whom all correspondence should be addressed.

Superficial dorsal muscle fibers were selected for electrophysiological study. Intracellular recording of MEPSPs was performed with conventional microelectrodes filled with 4 M potassium acetate with resistance between 2–7 MΩ. Animals less than 8 cm long were preferred because of the higher input resistance of their muscle fibers. MEPSPs were displayed on an oscilloscope screen and photographed on moving film. Records were divided into intervals 30 s long, and the frequency of spontaneous release was expressed as number/second.

Solution changes required about one min through a continuous perfusion system. All experiments were performed at room temperature in order to avoid temperature variations during solution changes.

The crayfish ringer contained 195 mM NaCl, 5.4 mM KCl, 13.5 mM CaCl$_2$, 2.6 mM MgCl$_2$, and 10 mM Hepes. The pH was adjusted to 7.3 through the addition of a few drops of NaOH. Calcium-free solutions had 5 mM EGTA, and magnesium was substituted for calcium. In some experiments, solutions contained 13.5 mM cobalt in place of calcium. Depolarizing solutions contained 22 mM KCl instead of the usual concentration.

RESULTS

Elevating the osmolarity of the external solution with the addition of 300 mM sucrose caused a 3–10-fold increase in the frequency of spontaneous release, as reported previously (van der Kloot and Kita, 1974). A similar rise occurred in the absence of external calcium [Fig. 1(A), sector C]. When the membrane was depolarized, the MEPSP frequency was reduced [Fig. 1(A), sector D]. Shimoni et al. (1977) interpreted such an observation in the frog neuromuscular junction as indicating a loss of internal calcium flowing through open voltage-dependent membrane channels toward the calcium-free medium, where the cation is sequestered by EGTA.

This interpretation implies that an increased internal calcium concentration is the cause of hyperosmotic neurosecretion, and it is based on the assumption that the experimental conditions are causing an outflow of calcium from the terminal. We decided to test this interpretation, substituting cobalt for calcium in the external solution. Cobalt is generally used to prevent an inflow of calcium from the external medium, but we thought that it could also reduce the rate of calcium outflow. The effects of cobalt are shown in Fig. 1(B), where it is possible to see (sector D) that depolarization of the membrane no longer causes a drastic reduction in MEPSP frequency. Effects on the presence and absence of cobalt on a single preparation are shown in Fig. 2 and are evident comparing sectors G with C and G with H.

DISCUSSION

Many theories have been proposed to explain the rise in spontaneous release following an increase in external osmotic pressure. The most convincing, however, seems to be the elevated calcium hypothesis (Shimoni et al., 1977). According to this explanation an increase in spontaneous transmitter release results from an increase of calcium concentration inside the nerve terminal. The effect of osmolarity would then be to change, somehow, the resting calcium concentration. This could happen in different ways. For example, it could cause the release of calcium from intracellular stores, enhance the influx of the cation through the membrane or block its efflux, or act by shrinking the terminal volume.

Muchnik and Venosa (1969) suggested that the hyperosmotic response is caused by an increase in internal sodium concentration. It has in fact been shown in squid that increasing [Na]$_i$ or decreasing [Na]$_o$ causes a larger
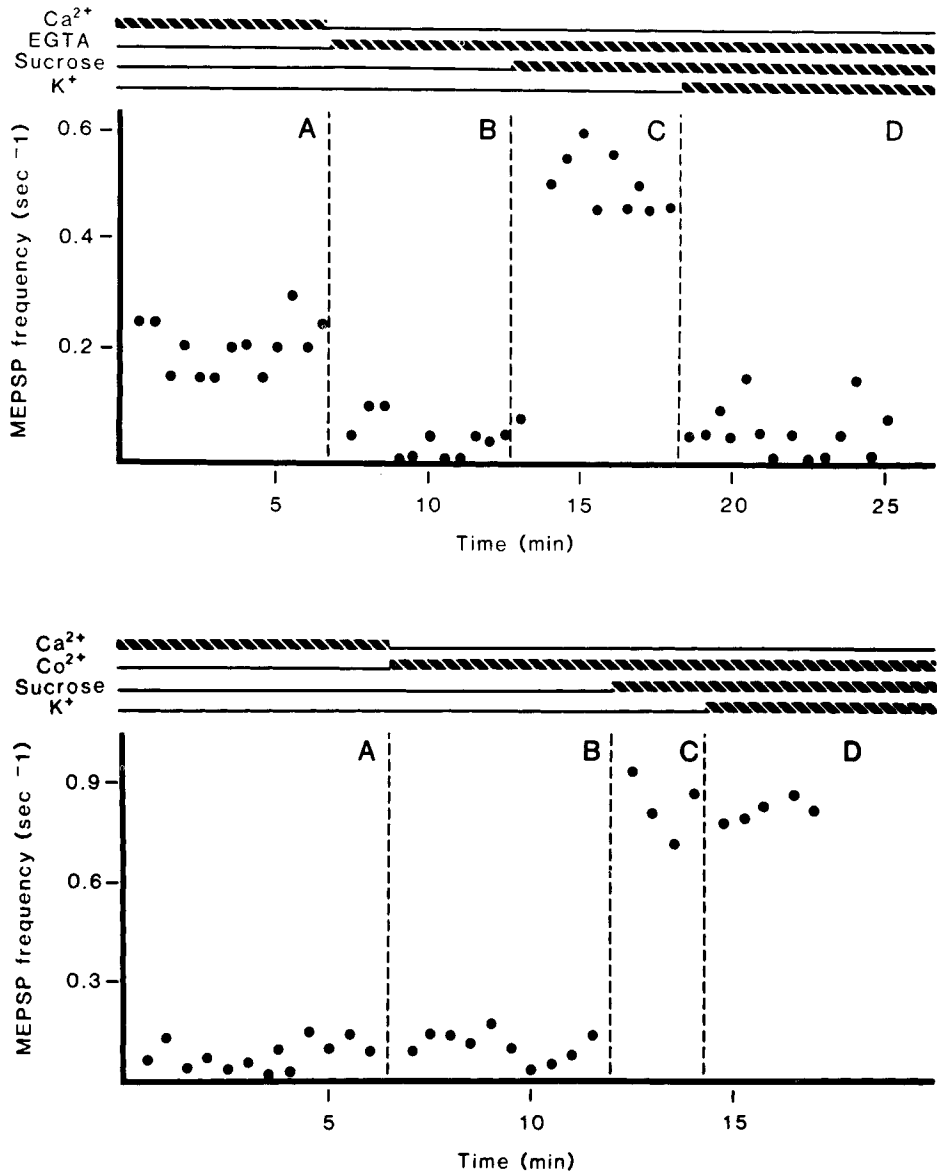
Fig. 1. **Top**: effect of depolarization on spontaneous transmitter release in conditons of hyperosmosis and absence of calcium. Removing calcium from the medium causes a slight decrease in MEPSP frequency (sector B). The addition of sucrose increases the osmolarity of the external solution and leads to a rise in frequency of spontaneous release (sector C). A higher concentration of external potassium causes a depolarization of the muscle fiber (about 20 m V) and presumably has the same effect on the nerve terminal. During the depolarizing condition the MEPSP frequency is sharply reduced. **Bottom**: effect of cobalt. The previous experiment is repeated on a different preparation, and cobalt is substituted for calcium. The resting frequency of spontaneous release is again increased when sucrose is added to the bath (sector C) but this time it remains elevated even when the membrane is depolarized (sector D). Each experiment has been repeated on five different preparations.
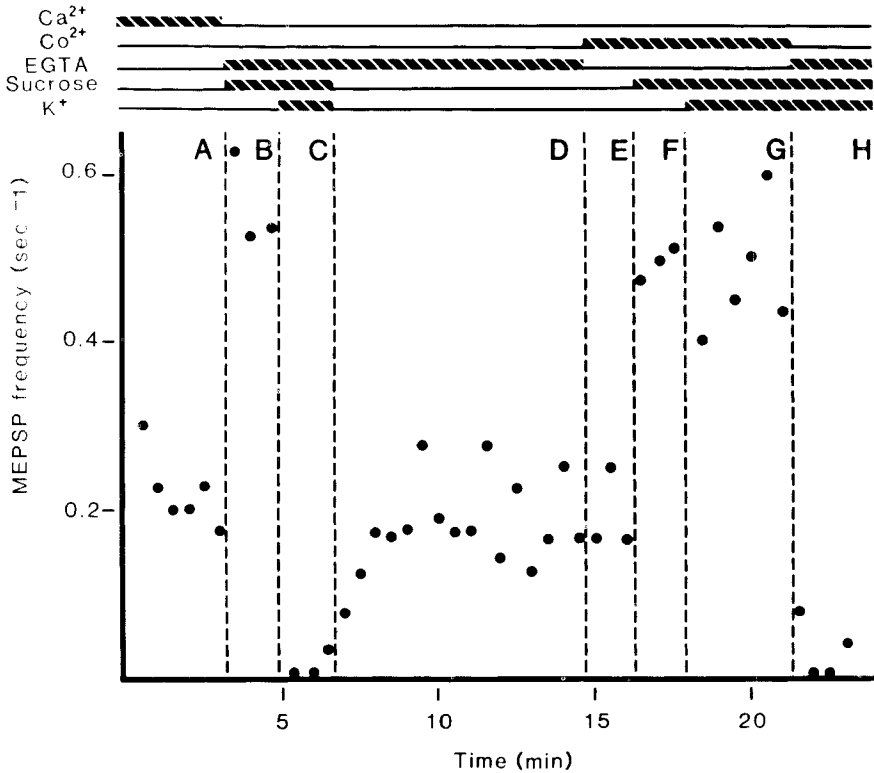
Fig. 2. The effect of depolarization in hyperosmotic calcium-free media in the presence and absence of cobalt tested in a single fiber. A sharp drop of MEPSP frequency is noted during depolarization in a hyperosmotic free-calcium medium (sector C). Sucrose is removed and the fiber is allowed to rest for 8 min in order to have full restoration of normal osmotic conditions (sector D). Cobalt is substituted for magnesium in the calcium-free solution (sector E). The frequency of spontaneous release is increased through the addition of sucrose to the medium, and it remains high during potassium-induced depolarization (sector G). It finally drops when cobalt is removed from the bath (sector H).

calcium influx owing to a sodium-calcium exchange pump in the plasmolemma (Baker et al., 1969). This observation, however, cannot explain the higher frequency of spontaneous release observed during hyperosmosis, since a hyperosmotic neurosecretion occurs even in the absence of external calcium (Blioch et al., 1968).

Rahamimoff et al. (1976) proposed the mitochondrion as a possible source of calcium for increasing its internal concentration in frog motor nerve terminals. This hypothesis is supported by the finding that an increase in osmolarity around liver mitochondria inhibits calcium uptake (Scarpa and Azzone, 1968).

The linkage between osmolarity and increase of internal calcium concentration could be a mechanical one. Assuming that the nerve terminal behaves as an osmometer, a doubling in osmolarity of the external solution would cause a reduction of the terminal volume to half. This shrinkage, in turn, would double the internal ionic concentrations. If transmitter release depends on a high power of internal calcium, this could easily account for the approximately 10-fold increase in MEPSP frequency observed at the crayfish neuromuscular junction.

The main argument raised against the elevated calcium theory is that hyperosmosis increases the frequency of spontaneous release without affecting the time course of phasic release by an action potential (Kita and van der Kloot, 1977). In other words: how can the internal calcium concentration be elevated enough to raise MEPSP frequency but not sufficient to prolong the duration of an excitatory postsynaptic potential?

Modeling studies of presynaptic calcium diffusion (Zucker and Stockbridge, 1983; Fogelson and Zucker, 1985) can answer the question. According to these simulations, the release of transmitter is not terminated by a transport or sequestering mechanism but mainly by the diffusion of calcium away from the release sites inside the terminal. The sharp rise in calcium near the release sites during a spike, which controls the release, is almost totally dissipated in about 0.5 msec, terminating in this way the release of neurotransmitter. This behavior is quite different from that of total calcium in the cytoplasm. The simulations also demonstrate that an elevated resting calcium, although increasing MEPSP frequency, would not affect the time course of phasic transmitter release evoked by an action potential.

Many experimental results favor the interpretation of hyperosmotic neurosecretion as due to an elevation of internal calcium, even though direct evidence for it is still missing. Blioch et al. (1968) showed that external calcium plays no direct role in this mechanism, and Shimoni et al. (1977) demonstrated that, in a condition of reversed calcium-concentration gradient and increased calcium conductance (g_{Ca}), there is a decrease in hyperosmotic secretion. This result suggests that when internal calcium is lowered, hyperosmotic secretion decreases. Our work reinforces this interpretation, showing that this remarkable decrease in MEPSP frequency can be reduced or abolished by external cobalt.

Divalent cations have recently been shown to block Na/Ca exchange in neurons (Requena et al., 1985). We considered whether the decrease in MEPSP frequency on depolarization in hyperosmotic medium could be due to an effect on the Na/Ca pump which is blocked by cobalt. The following argument excludes this possibility: since three or more sodium ions are pumped for each calcium ion (Baker et al., 1969), the Na/Ca exchange pump normally extrudes calcium with a net inward current due to sodium influx. Membrane depolarization, therefore, retards the operation of this pump and reduces calcium efflux (DiPolo et al., 1985). This in turn should cause an increased accumulation of internal calcium and a subsequent rise in MEPSP frequency. However, in a hyperosmotic calcium-free medium, depolarization causes a decrease, not an increase, in spontaneous release. Therefore, either changes in transmitter release following osmotic variations are not mediated by the Na/Ca pump, or such effects are overwhelmed by the opening of calcium channels. In any case cobalt has an effect other than blocking the pump.

In summary, experimental work indicates that an increase in MEPSP frequency during hyperosmosis is caused by an elevation in internal calcium and that it can be counteracted when external conditions allow internal calcium to flow out through membrane channels if these are not blocked by cobalt.

Finally, our results show that cobalt, a cation known to prevent the influx of calcium, seems also to reduce the outflow of calcium from the terminal. It is important to note that this conclusion arises only from the indirect

measurement of calcium movement through the membrane, but it invites further consideration of this aspect of cobalt as a calcium-channel blocker.

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