Monensin can transport calcium across cell membranes in a sodium independent fashion in the crayfish *Procambarus clarkii*

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Monensin, a Na^+-selective ionophore, enhances transmitter release when applied to crustacean and frog neuromuscular junctions. Monensin is believed to raise intracellular sodium ($[Na^+]_i$) which in turn elevates intracellular calcium ($[Ca^{2+}]_i$). Using the fluorescent indicator fura-2, we measured $[Ca^{2+}]_i$ in crayfish *Procambarus clarkii* presynaptic terminals during monensin application in normal Ringer, zero-calcium Ringer and zero-sodium Ringer to determine if $[Ca^{2+}]_i$ increases with monensin application and if so by what mechanism. In normal Ringer, monensin, 10 μM and 100 μM, elevated $[Ca^{2+}]_i$ by 440 nM and 7 μM respectively. This rise in $[Ca^{2+}]_i$ was dependent on external calcium, as $[Ca^{2+}]_i$ did not increase in zero-calcium Ringer. However, in a zero-sodium Ringer, monensin (10 μM) elevated $[Ca^{2+}]_i$ by 370 nM. It is important to recognize that monensin, thought to be a sodium-selective ionophore, can transport calcium across the cytoplasmic membrane in a sodium-independent manner.

Due to the possible link between posttetanic potentiation (PTP) and the accumulation of $[Na^+]_i$ [10] there has been interest in the various pharmacological methods available which elevate $[Na^+]_i$ in cells. One of the methods which has been widely used is the Na^+-selective ionophore monensin [1, 3, 4, 9]. Monensin is thought to act as a sodium ion/hydrogen ion antiport, exchanging one sodium in for one proton out [3].

Monensin has been applied to the crustacean and frog neuromuscular junction where enhancement of transmitter release was recorded [1, 4]. Since monensin was able to enhance transmitter release in the absence of external calcium by increasing the frequency of spontaneous release [4] as well as enhance evoked release upon restoration of normal calcium [1], it was concluded monensin must act by elevating $[Na^+]_i$ which in turn elevates $[Ca^{2+}]_i$.

Various mechanisms by which intracellular sodium could enhance transmitter release have been proposed. Sodium ion accumulation may elevate calcium either through release of calcium from intracellular stores, by entry of calcium from the external medium through Na/

Ca exchange or by slowing extrusion of accumulated calcium due to a reduction in the sodium gradient which drives Na/Ca exchange.

To test the mechanism by which monensin presumably increases the resting $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ in presynaptic terminals of crayfish opener muscles, using the fluorescent indicator fura-2. We measured $[Ca^{2+}]_i$ in the presence of monensin under conditions where (1) external calcium was removed, (2) external sodium was eliminated, and (3) normal Ringer was present.

All experiments were performed on the opener muscle of the first walking leg of crayfish, *Procambarus clarkii*. The injection of fura-2 into the excitatory axon and imaging of the presynaptic terminals was identical to the methods described in [6].

Normal Van Harraveld's solution contained (in mM), 195 NaCl, 13.5 $CaCl_2$, 5.4 KCl, 2.6 $MgCl_2$, and 10 Na-HEPES at pH 7.4. The EGTA Ringer contained 185 NaCl, 5.4 KCl, 30 mM $MgCl_2$, 2 Na_2EGTA, and 10 Na-HEPES at pH 7.4. Zero-sodium choline Ringer contained choline in place of sodium, plus 13.5 $CaCl_2$, 2.6 $MgCl_2$, and 6 K-HEPES. In addition, a Tris Ringer, in which Tris was substituted for sodium, and an *N*-methyl-D-glucamine Ringer, in which glucamine was substituted for sodium, were used in these experiments. Monensin was obtained from Sigma and highly purified monensin from Cal Biochem; stock solutions were made by dissolving in ethanol. A final concentration was obtained by

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diluting in the appropriate normal Van Harraveld's solution with ethanol being $\leq 0.1\%$.

The temperature of the preparation was kept at $18 \pm 2°C$ with a stage-mounted peltier device. A gravity perfusion system controlled by a solenoid 4-way valve (General Valve, Series 18, Fairfield NJ) was used to switch between normal and modified salines. An inflow was positioned above the surface of the opener muscle and solutions flowed under the 40× water immersion lens. However, to ensure complete removal of calcium from the bath, the objective was lifted out of the bath and the surface washed vigorously with a syringe which had a 1 mm-diameter plastic tube attached to the tip, while also continuing to perfuse with the gravity system. A perfusion time of about 45 to 60 minutes and several vigorous rinses were needed to eliminate calcium influx with zero-calcium EGTA Ringer.

To determine $[Ca^{2+}]_i$ from stored images, circular masks, encompassing 100–300 pixels, were positioned over terminals and the $[Ca^{2+}]_i$ was spatially averaged.

It is thought that elevation of $[Na^+]_i$ can lead to an increase in $[Ca^{2+}]_i$ [8]. Two ways in which sodium accumulation could increase resting $[Ca^{2+}]_i$ is by releasing calcium from internal stores or by calcium influx from the external medium via alteration of the Na/Ca exchange. As a way to test these two possibilities we tried using the Na^+-selective ionophore, monensin [7].

Addition of monensin (10 μM) in an EGTA Ringer (zero-calcium, 2 mM EGTA and 30 mM Mg^{2+}) to the crayfish presynaptic terminals did not increase $[Ca^{2+}]_i$, as shown in Fig. 1. This result is typical of 6 terminals measured in 3 preparations, having a mean $\pm$ S.E.M. equal to 0 ± 10 nM. As shown in Fig. 1, upon wash-in of normal Ringer the $[Ca^{2+}]_i$ increased by approximately 80 nM, but with addition of monensin (10 μM) in normal Ringer the $[Ca^{2+}]_i$ was raised by 500 nM, with a mean $\pm$ S.E.M. of 440 ± 40 nM in 6 terminals in 3 preparations, designated N=6,3. It routinely took 3 to 4 minutes to see an increase in $[Ca^{2+}]_i$ following addition of monensin, with a peak response occurring in about 10 minutes. Upon wash-out of monensin with normal Van Harraveld's solution, the $[Ca^{2+}]_i$ returned to normal in approximately 1 h. (Fig. 1). In 2 terminals measured in 1 preparation, a *larger* dose of monensin (100 μM) was used. The $[Ca^{2+}]_i$ increased in a zero-calcium EGTA bathing solution by 150 nM. With addition of monensin in normal saline, the $[Ca^{2+}]_i$ increased to $7 \pm 1.4 \mu$M as measured in the 2 terminals. The relatively small elevation of $[Ca^{2+}]_i$ seen with a *large* dose of monensin (100 μM) in zero-calcium was probably due to insufficient removal of calcium from the external medium, as it has been shown external calcium surrounding nerve terminals is difficult to remove [5]. The monensin effect was not attributable to the ethanol
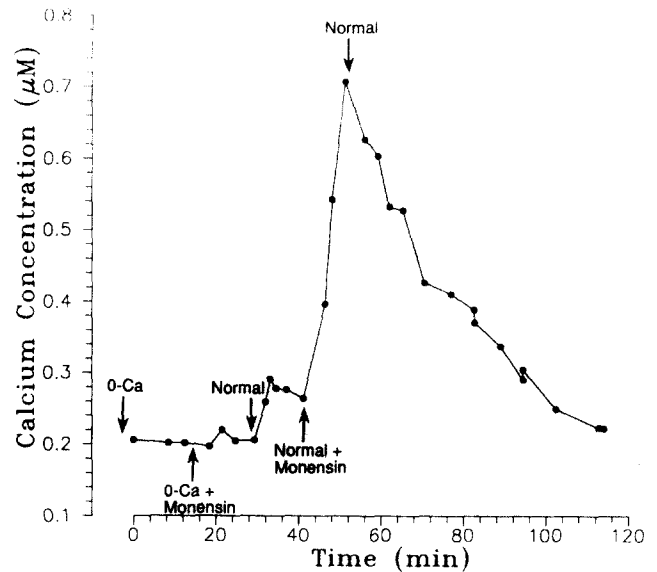

Fig. 1. Effects of monensin on $[Ca^{2+}]_i$ in calcium-free and normal Ringer. $[Ca^{2+}]_i$ was measured in zero-calcium Ringer prior to monensin application. Monensin (10 μM), added to calcium-free Ringer, produced no change in $[Ca^{2+}]_i$. Upon washing out the monensin with normal Ringer, $[Ca^{2+}]_i$ increased by about 80 nM. Monensin in normal Ringer was then added and $[Ca^{2+}]_i$ increased by 500 nM, taking approximately 10 min to reach a peak response. $[Ca^{2+}]_i$ returned to control values over a one-hour period, with continuous perfusion of normal Ringer.

used to dissolve the monensin, as addition of the ethanol alone (0.1%) in normal Van Harraveld's solution produced no increase in $[Ca^{2+}]_i$ over an equivalent time period to that of monensin exposure. These results suggest sodium ion accumulation, as a result of monensin application, elevates $[Ca^{2+}]_i$ most likely through an effect on Na/Ca exchange, since $[Ca^{2+}]_i$ increases only when external calcium is present.

To test the possibility that monensin was depolarizing the presynaptic membrane and allowing calcium entry through voltage-dependent calcium channels, the presynaptic membrane potential was monitored during monensin application. There was no recorded change in the presynaptic membrane potential during 10 μM monensin exposure, but the $[Ca^{2+}]_i$ increased by 270 and 210 nM, as measured in 2 terminals. The action potential amplitude decreased by 5 to 6 mV, probably due to $[Na^+]_i$ accumulation reducing the sodium gradient, and the action potential broadened by 1 ms. Recently, a study reported a 20 to 30 mV hyperpolarization in response to 20 μM monensin application in neuroblastoma-glioma hybrid cells and attributed this to an increase in Na/K exchange activity, as the hyperpolarization was blocked by ouabain [3]. Because we observed no change in membrane potential, the possibility monensin was admitting calcium by activating voltage-dependent calcium channels was ruled out.

To be certain monensin's effect of elevating $[Ca^{2+}]_i$ was by accumulation of $[Na^+]_i$, monensin was added to various zero-sodium Ringers, where choline, Tris, or N-methyl-D-glucamine were substituted for sodium. Fig. 2 shows the effect of monensin (10 μM) in Tris Ringer on the $[Ca^{2+}]_i$. The preparation was washed in Tris Ringer for 50 min and the zero-sodium monensin solution was added. Intracellular calcium concentration increased by 190 nM in one terminal and 140 nM in a second terminal. The zero-sodium monensin solution was washed out and the $[Ca^{2+}]_i$ returned to normal in approximately 5 min. A normal Ringer monensin solution was added and $[Ca^{2+}]_i$ increased to 260 and 270 nM in the 2 terminals. The intracellular calcium returned to close to normal in approximately 23 min upon addition of normal Ringer. This experiment was repeated in 5 preparations, 11 terminals; three times in choline Ringer, once in Tris Ringer and once in glucamine Ringer to assure the molecule chosen for sodium substitution did not affect the increase in $[Ca^{2+}]_i$. The results were very similar in all three solutions with a mean increase in $[Ca^{2+}]_i$ upon addition of monensin equal to 370±40 nM. The increase in $[Ca^{2+}]_i$ when monensin (10 μM) was added to normal Ringer containing sodium ions was 360±30 nM measured in 17 terminals, 9 preparations. The mean increase in $[Ca^{2+}]_i$ did not differ between monensin application in a sodium containing Ringer or a zero-sodium substituted Ringer, even though it must be taken into account a second application of monensin may not be as effective.

Since monensin did not appear to be dependent on extracellular sodium to raise $[Ca^{2+}]_i$, we thought perhaps an impurity in the monensin was accounting for this result. We therefore tried the highly purified form of monensin at a dose of 10 μM, added to a zero-sodium choline Ringer. Surprisingly, the $[Ca^{2+}]_i$ as measured in 3 terminals in 1 preparation, increased to 15±5 μM. It appeared the highly purified form of monensin (at the same dose) was much more effective in raising $[Ca^{2+}]_i$ with no sodium in the external medium, than the type thought to be less pure.

Our experiments suggest monensin is capable of admitting calcium in a sodium-independent manner, even though monensin has been reported to have a 350,000 sodium:calcium selectivity ratio [7]. Previous experiments at the crab neuromuscular junction have shown, however, a dependence of monensin on extracellular sodium. Atwood and colleagues [1] lowered the external sodium to 33% of normal and showed under these conditions, in the presence of monensin, transmitter release (measured as the excitatory junctional potential (EJP)) increased by about 30%. When normal sodium was returned, plus monensin, the EJP amplitude increased another 40%. This additional enhancement of EJP ampli-
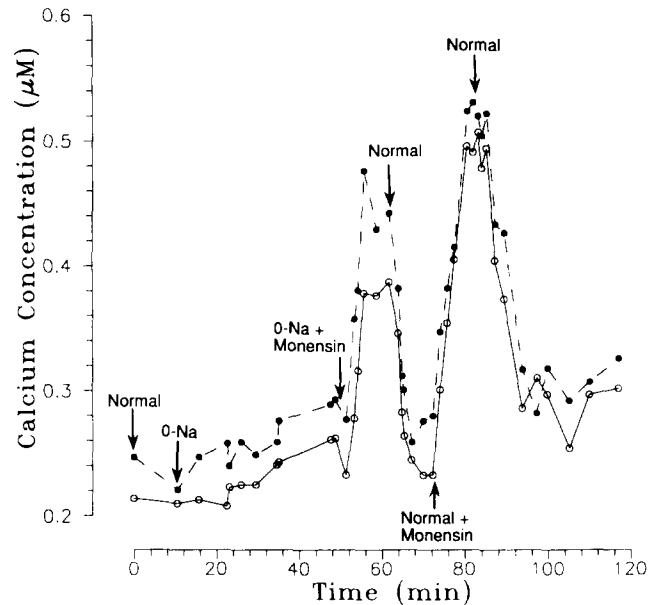

Fig. 2. Effect of monensin on $[Ca^{2+}]_i$ in a sodium-free and normal sodium-containing Ringer. $[Ca^{2+}]_i$ was measured in 2 terminals shown by a dashed line and a solid line. $[Ca^{2+}]_i$ was measured prior to sodium-free Ringer perfusion. The zero-sodium Ringer was perfused for 50 min before monensin was added. Upon addition of monensin (10 μM) in sodium-free Ringer, the $[Ca^{2+}]_i$ increased by 190 nM in 1 terminal and 140 nM in the other. A peak response was reached in 5 min. Monensin was washed out by perfusing with normal Ringer and $[Ca^{2+}]_i$ values were back to control in about 5 min. Monensin (10 μM), added to normal sodium-containing Ringer, increased $[Ca^{2+}]_i$ by 260 nM and 270 nM in the 2 terminals. The decline of $[Ca^{2+}]_i$ with perfusion of normal Ringer took over 20 min to approach resting values.

tude was seen only when monensin was added to normal Ringer, suggesting it was a presynaptic sodium accumulation effect brought about by monensin and not the result of the sodium-dependence of the postsynaptic current. If monensin is not working via a presynaptic sodium-dependent mechanism leading to an increase in $[Ca^{2+}]_i$, how can this result be explained? The change in $[Ca^{2+}]_i$ that would have to occur to account for a 50% enhancement in EJP amplitude at the crayfish neuromuscular junction can be calculated [2]. A rise of $[Ca^{2+}]_i$ by approximately 20 nM would increase the EJP amplitude by 50%, recognizing that the relationship between $[Ca^{2+}]_i$ and EJPs was measured at the crayfish neuromuscular junction, not the crab. Therefore, the EJP enhancement recorded in a low-sodium monensin solution at the crab neuromuscular junction might be the result of approximately 15 nM increase in $[Ca^{2+}]_i$, while a further 20 nM increase would account for the enhancement in monensin plus normal sodium [1]. In 3 preparations out of 5, the zero-sodium monensin experiments did not elevate $[Ca^{2+}]_i$ as high as in the normal sodium medium, as illustrated in Fig. 2. The difference of monensin's effectiveness in increasing $[Ca^{2+}]_i$ to a higher value in a sodium-

containing medium may explain the increased enhancement seen [1] and may suggest monensin works in a sodium-dependent manner, when external sodium is available. In one preparation, the zero-sodium monensin solution increased $[Ca^{2+}]_i$ more (as measured in 2 terminals, $[Ca^{2+}]_i$ was 510 and 600 nM compared to 240 and 270 nM for the normal sodium monensin solution). It is possible that a second application of monensin may not be as effective as the first, which was reported at the crab neuromuscular junction [1] in regard to enhancement of the EJP. This would explain the higher increase in $[Ca^{2+}]_i$ sometimes seen in a monensin zero-sodium solution over subsequent exposure to monensin in normal sodium and does not suggest monensin is more effective when sodium is absent. The important point is, not whether $[Ca^{2+}]_i$ reached a higher value in a Na^+-free or a normal sodium solution, but that $[Ca^{2+}]_i$ is elevated under both conditions. When the highly purified form of monensin was used when external sodium was absent, it was also quite capable of raising $[Ca^{2+}]_i$ to the micromolar range. Because $[Ca^{2+}]_i$ went so high in this preparation, another application of normal Ringer plus monensin was not done.

Monensin added to a zero-calcium, EGTA Ringer increased the frequency of miniature end plate potentials at the frog neuromuscular junction [4]. We found with vigorous perfusion, that monensin had no effect on elevating the $[Ca^{2+}]_i$ when external calcium was removed. As discussed previously [5], it is very difficult to eliminate calcium from the external medium, even when EGTA is used. Monensin's elevation of $[Ca^{2+}]_i$ appears to be dependent on external calcium primarily by directly transporting it across the cell membrane.

It is possible the monensin obtained from both Sigma and Cal Biochem were contaminated with other compounds which led to the elevation of $[Ca^{2+}]_i$. Whatever the explanation, it is important to recognize that commercially available monensin, which is thought to be a Na^+-selective ionophore, may also be transporting other ions, specifically calcium, across the cytoplasmic membrane. This, as a result, may lead to the misinterpretation of experimental results.

1 Atwood, H.L., Charlton, M.P. and Thompson, C.S., Neuromuscular transmission in crustaceans is enhanced by a sodium ionophore, monensin, and by prolonged stimulation. J. Physiol., 335 (1983) 179–195.
2 Delaney, K.R., Zucker, R.S. and Tank, D.W., Calcium in motor nerve terminals associated with posttetanic potentiation, J. Neurosci., 9 (1989) 3558–3567.
3 Lichtshtein, D., Dunlop, K., Kaback, H.R. and Blume, A.J., Mechanism of monensin-induced hyperpolarization of neuroblastoma-glioma hybrid NG108-15, Proc. Natl. Acad. Sci. USA, 76 (1979) 2580–2584.
4 Meiri, H., Erulkar, S.D., Lerman, T. and Rahamimoff, R.,The action of the sodium ionophore, monensin, on transmitter release at the frog neuromuscular junction, Brain Res., 204 (1981) 204–208.
5 Mulkey, R.M. and Zucker, R.S., Action potentials must admit calcium to evoke transmitter release, Nature, 350 (1991) 153–155.
6 Mulkey, R.M. and Zucker, R.S., Posttetanic potentiation at the crayfish neuromuscular junction is dependent on both intracellular calcium and sodium ion accumulation, J. Neurosci., in press.
7 Pressman, B.C., Biological applications of ionophores, Annu. Rev. Biochem., 45 (1976) 501–530.
8 Rahamimoff, R., Lev-Tov, A. and Meiri, H., Primary and secondary regulation of quantal transmitter release: calcium and sodium, J. Exp. Biol., 89 (1980) 5–18.
9 Smith, J.B. and Rozengurt, E., Serum stimulates the Na^+, K^+ pump in quiescent fibroblasts by increasing Na^+ entry, Proc. Natl. Acad. Sci. USA, 75 (1978) 5560–5564.
10 Wojtowicz, J.M. and Atwood, H.L., Presynaptic long-term facilitation at the crayfish neuromuscular junction: voltage-dependent and ion-dependent phases, J. Neurosci., 8 (1988) 4667–4674.