BRIEF COMMUNICATION

REGULATION OF NERVE TERMINAL CALCIUM CHANNEL SELECTIVITY BY A WEAK ACID SITE

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ABSTRACT The effects of low pH, and of alkaline earth cations, were examined on calcium uptake by pinched-off nerve terminals (synaptosomes). This uptake appears to be mediated by voltage-sensitive Ca channels (*J. Physiol.* **247**:617, 1975). Ca uptake was measured in low (5 mM) or high (77 mM) potassium media. The extra uptake promoted by depolarizing (K-rich) media was almost maximal at pH 7.5, and decreased as the pH was lowered. Data relating depolarization-induced ^{45}Ca uptake to pH fit a titration curve with a $pK_a \sim 6$. Experiments in which Ca concentration and pH were both varied indicated that Ca^{2+} and H^+ compete for a common binding site. Inhibition of depolarization-induced ^{45}Ca uptake by the alkaline earth cations was studied to determine the apparent binding sequence for these cations in the Ca channel: Ca > Sr > Ba > Mg. This sequence resembles that observed for block of Ca channels in other preparations. The apparent binding sequence of the alkaline earth cations and the apparent pK_a (~ 6) of the Ca-binding site indicate that the Ca channel is a "high field strength" system. Protonation of a Ca channel binding site could explain the inhibitory effect of low pH on Ca-dependent neurotransmitter release (cf. Del Castillo et al., *J. Cell. Comp. Physiol.* **59**:35, 1962).

INTRODUCTION

Divalent cation-selective channels located at presynaptic nerve terminals (Katz and Miledi, 1969) play a crucial role in triggering neurotransmitter release (cf. Katz, 1969; Blaustein et al., 1977). The properties of these channels may be conveniently studied in pinched-off presynaptic terminals (synaptosomes) prepared from vertebrate brain homogenates. Synaptosomes have been shown to retain many of the functional properties of intact terminals (cf. Bradford, 1975; Blaustein et al., 1977). In particular, one mode of Ca entry in synaptosomes appears to be mediated by voltage-sensitive Ca channels that are blocked by Mn^{2+} and La^{3+} (Blaustein, 1975; see Blaustein and Oborn, 1975 for a discussion of modes of Ca uptake in synaptosomes).

There is evidence that the ionic selectivity of voltage-sensitive Ca channels is determined by a binding site in the channel (Hagiwara, 1975; Akaike et al., 1978). We report here that the binding site associated with the Ca channel of synaptosomes has a $pK_a \sim 6$. The sequence for binding of alkaline earth cations to this site, Ca > Sr > Ba > Mg, is the same as that seen in the Ca channels of other preparations (Hagiwara, 1975; Akaike et al., 1978). A preliminary

report of some of our findings has been communicated to the American Physiological Society (Nachshen and Blaustein, 1978).

METHODS

Synaptosomes were prepared from rat brains by the method of Hajos (1975). The terminals in the 0.8-M fraction from the sucrose gradient (Hajos, 1975) were equilibrated by the addition of 2.5 vol of ice-cold equilibration salt solution (Na + 5K) containing (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1.4; $CaCl_2$, 1.2; Tris-maleate, 10, adjusted to pH 7.5 at 25°C with NaOH. The synaptosomes were centrifuged and the pellet was resuspended in cold Na + 5K, but with $CaCl_2$ reduced to 0.06 mM unless otherwise noted (see legends to Figs. 2 and 3). The suspension was then preincubated for 20–35 min at 30°C. Test incubations were carried out by adding 0.2 ml of the preincubated synaptosome suspension to 0.2 ml of Na + 5K containing ^{45}Ca, or to a similar solution in which K was substituted isosmotically for all of the Na so that the final K concentration was 77.5 mM (= K-rich, or depolarizing, solution). The pH of the ^{45}Ca-containing solutions was adjusted with HCl or NaOH to give the desired value during the incubation period. ^{45}Ca uptake was stopped after 120 s (Fig. 1) or 10 s (Figs. 2 and 3) by diluting the suspensions (0.4 ml) with 4.5 ml of ice-cold Na + 5K. The diluted suspensions were immediately filtered by suction through Whatman glass fiber filters (Whatman Inc., Clifton, N.J.) (2.4-cm diam, GF/C). The filters were washed twice with 4.5-ml aliquots of ice-cold Na + 5K; the ^{45}Ca content of the material trapped on the filters was determined by liquid scintillation spectrometry. Protein in the synaptosome suspensions was determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

The extra ("stimulated") ^{45}Ca uptake, promoted by the K-rich ("depolarizing") solution, is mediated by voltage-sensitive Ca channels (Blaustein, 1975). The magnitude of this extra uptake of ^{45}Ca, induced by the K-rich solution, decreased as the pH of the external medium was lowered. Stimulated Ca uptake is plotted as a function of external pH in Fig. 1; the data fit the titration curve for a weak acid with a $pK_a \sim 6$. This suggests that an acidic group is associated with the Ca channel, and that it must be dissociated to allow Ca^{2+} ions to pass.

Control experiments (data not shown) were carried out to determine whether or not the drop in pH reduces potassium's effectiveness as a depolarizing agent. We used the voltage-sensitive fluorescent dye, 3,3′-dipentyl 2,2′-oxacarbocyanine (Blaustein and Goldring, 1975) to monitor the effects of changing the external pH on synaptosome membrane potential. When the pH of the medium (normally 7.5) was reduced to 5.8, or increased to 8.5, there was no effect on the change in dye fluorescence induced by depolarizing the synaptosomes in high-K solution. This is evidence that the change in pH does not significantly affect the K-induced membrane depolarization.

In other experiments, K-stimulated ^{45}Ca uptake was measured at both normal and low pH, at a variety of Ca concentrations. As illustrated by the double reciprocal plot of Fig. 2, lowering the pH of the solution did not alter the maximal uptake predicted for saturating Ca concentrations (J_{Ca}^{max} in Eq. 3, Fig. 2), but it did increase the apparent affinity constant of the Ca-binding site for Ca (K_{Ca}). These results indicate that Ca^{2+} and H^+ may compete for a common binding site in the Ca channel. The apparent pK_a of the binding site in this

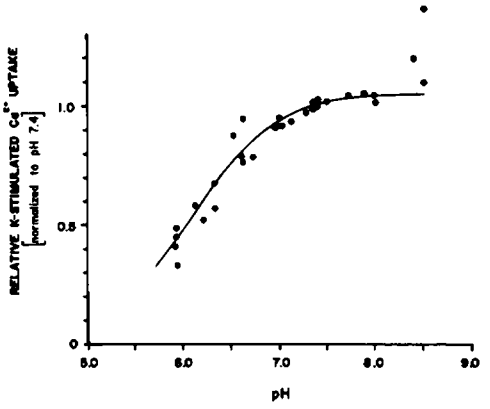

FIGURE 1 Effect of pH on K-stimulated ^{45}Ca uptake by synaptosomes. The terminals were incubated with ^{45}Ca for 2 min in solutions containing 60 μM Ca and either 5 mM K and 145 mM Na (control solution) or 77.5 mM K and 77.5 mM Na (depolarizing solution). Stimulated ^{45} Ca uptake values (depolarized minus control influxes) were normalized to the values at pH 7.4 (= 1.00%). The curve relating uptake to pH was fitted, by the method of least squares, to the equation:

$$J_{Ca} = J_{Ca}^{max}/(1 + [H^+]/K_H). \quad (1)$$

J_{Ca} is the stimulated Ca uptake at any pH, J_{Ca}^{max} is the maximal uptake (1.06%), $[H^+]$ is the proton concentration of the incubation solution, and $pK_a = -\log K_H = 6.08$. Data from six experiments are shown; each symbol indicates the mean of three determinations from one experiment. The two data points measured at pH 8.5 were not included in the computation of the best fit. The percent root mean square deviation for the estimate of pK_a was $\pm 5\%$. Estimates of K_H in the absence of divalent cations were made using the following equation:

$$K_H = \frac{K_H\,(app)}{1 + [I]/K_I}, \quad (2)$$

where K_H (app) is the value of K_H in the presence of divalent cation I, [I] is the concentration of I, and K_I is the half-saturation value of the site for I (taken from the data in the legend to Fig. 3). After correcting for the presence of Ca and Mg in the solution, pK_a was estimated as 6.3. Ca uptake from control solution (Na + 5K) averaged 0.83 $\pm$ 0.15 nmol/mg protein per 2 min. K-rich solutions stimulated Ca uptake 3–4-fold at pH 7.5.

experiment was 6.5, as calculated from Eq. 3 in the legend to Fig. 2. This value is in good agreement with the pK_a value (6.3) derived from the best fit to the data in Fig. 1, even though the incubation times were very different in the two sets of experiments (10 vs. 120 s). The fact that micromolar concentrations of Ca compete with protons in the presence of 1.4 mM Mg indicates that surface charge effects cannot account for the observed interaction between Ca and protons.

We also tested the inhibitory effects of various alkaline earth cations on K-stimulated ^{45}Ca uptake. As shown in Fig. 3, the sequence of apparent inhibitory constants (K_i's) for 50% block of ^{45}Ca uptake was: $K_{i(Ca)} < K_{i(Sr)} < K_{i(Ba)}$. A similar affinity sequence has been found for the Ca-channels in barnacle muscle (Hagiwara, 1975) and snail neurones (Akaike et al., 1978).

Eisenman (1961) has suggested that the relative affinities of a site for monovalent and divalent cations may be explained by the different coulombic forces exerted on the cations by water, on the one hand, and by the binding site, on the other. The strength of the coulombic interactions (i.e., the electrostatic field strength of the anionic binding site) would then control

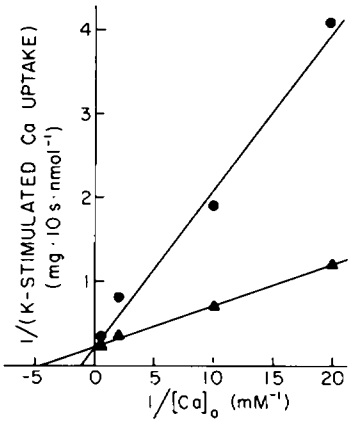

FIGURE 2 Effect of external Ca concentration on ^{45}Ca uptake by synaptosomes at pH 7.4 (▲) and pH 5.8 (●). $[Ca]_0$ in the equilibration and incubation solution was 20 μM, and incubation with ^{45}Ca was carried out for 10 s. Each symbol represents the difference between mean uptake from high K and from control (Na + 5K) solutions (three determinations, each). The data are shown in double-reciprocal (Lineweaver-Burke) plots. The regression lines for the K-stimulated Ca uptake at the two pH's were determined by the method of least squares. The lines fit the reciprocal form of the equation:

$$J_{Ca} = \frac{J_{Ca}^{max}}{1 + \frac{K_{Ca}}{K_H}\frac{[H^+]}{[Ca]_0} + \frac{K_{Ca}}{[Ca]_0}}, \quad (3)$$

where J_{Ca}, J_{Ca}^{max}, $[H^+]$, and K_H have the same meanings as in Fig. 1. $[Ca]_0$ is the external Ca concentration, and K_{Ca} is the apparent half-saturation constant for Ca. J_{Ca}^{max} (pH 7.4) was 4.75 nmol/mg protein per 10 s and J_{Ca}^{max} (pH 6.0) was 4.74 nmol/mg protein per 10 s. K_{Ca} was calculated as 165 μM, and pK_a was calculated as 6.5 after correction for the presence of 1.4 mM $MgCl_2$ (see Eq. 2 in the legend to Fig. 1).

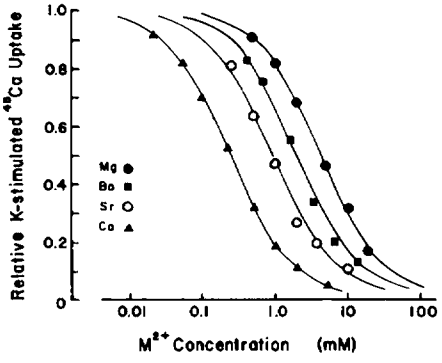

FIGURE 3 Inhibition of K-stimulated ^{45}Ca influx by alkaline earth cations. In these experiments synaptosomes were incubated with ^{45}Ca for 10 s. In experiments where $[Ca]_0$ was varied, the concentration of Mg was 1.4 mM. In all other experiments, $[Ca]_0$ was 20 μM. In experiments using Sr and Ba, $[Mg]_0$ was 0.5 mM. Alterations in the concentration of various divalent cations were balanced by adding equiosmolar concentrations of choline chloride to the control solutions. The solid lines are least-square fits to an equation having the same form as Eq. 1 (in the legend to Fig. 1). The curves have been normalized so that $J_{Ca} = 1.0$ when the concentration of the inhibition divalent cation is zero. The half-saturation values of the site, after correction for the presence of Ca and Mg (see Eq. 2 in the legend to Fig. 1), were (mM): $K_{Ca} = 0.15 \pm 0.01$; $K_{Sr} = 0.89 \pm 0.21$; $K_{Ba} = 1.56 \pm 0.08$; $K_{Mg} = 3.34 \pm 0.54$ (mean ± SEM, 4 experiments for each mean determination).

the selectivity sequence (Eisenman, 1961). The observed binding sequence of the alkaline earth cations for the Ca channel (Ca > Sr > Ba), and the relatively high pK_a (6.3) of the binding site, both suggest that the Ca channel is a high field strength system (cf. Diamond and Wright, 1969; Hille, 1975).

It should be noted, however, that in a high field strength system the predicted affinity for Mg is greater than for Ca (Diamond and Wright, 1969). But in fact, Mg is relatively ineffective as a blocker of Ca influx in synaptosomes (Fig. 3) and other preparations (Hagiwara, 1975; Akaike et al., 1978). Moreover, Mg does not carry significant inward current through the Ca channels of barnacle muscle (Hagiwara, 1975) and snail neurons (Akaike et al., 1978). It seems possible that, for divalent cations with radii much smaller than calcium's, the replacement of water from the primary hydration shell, by coordinating groups in the channel, may be energetically unfavorable (Urry, 1978).

Truesdell and Christ (1963) have proposed that at least two closely spaced binding sites are required to obtain strong selectivity of divalent over monovalent cations (Truesdell and Christ, 1963). Alternatively, Renugopalakrishnan and Urry (1978) have suggested that under certain conditions this requirement does not strictly hold. Our pH titration data do not demonstrate a second binding site at the Ca channel. However, if a second charged group does play a role in divalent ion selectivity, this group might be strongly acidic, thereby remaining dissociated in the pH range we examined.

Neurotransmitter release, at the frog neuromuscular junction, is reduced by lowering the pH of the bathing medium (Del Castillo et al., 1962), and it has been suggested that this reduction is caused by protonation of an acidic site (with a $pK_a \sim 5.7$) that blocks Ca influx into the nerve terminal (Landau and Nachshen, 1975). The present study provides direct evidence for such a mechanism in synaptosomes. The chemical nature of this site is, as yet, unknown; there are, however, few dissociable groups with a $pK_a \sim 6.3$. One such group is imidazole, with a pK_a in the range of 5.6–7.0 (Edsall and Wyman, 1958). However, considerable caution should be exercised in attempting to identify the binding site solely by its apparent pK_a insofar as this value could be sensitive to the microenvironment of the site. For instance, the relative position of the site with respect to the electrical field across the membrane could influence the observed pK_a (e.g., Woodhull, 1973). It may be possible, by the use of appropriate affinity labeling techniques, to establish the identity of the weak acid site, which seems to be important for regulating Ca-channel selectivity.

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