Effects of membrane depolarization on intracellular calcium in single nerve terminals

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The $[Ca^{2+}]_i$ of individual neurosecretory nerve terminals loaded with the fluorescent probe fura-2 was monitored during depolarizing stimuli and in the presence of substances known to induce or block neurohormone release. Induction of membrane depolarization with elevated $[K^+]$ or veratridine led to a rapid increase in $[Ca^{2+}]_i$ that was sensitive to block by substances which block voltage-sensitive L-type Ca^{2+} channels such as the dihydropyridine nicardipine and by D-888. Relaxin, cholecystokinin and enkephalin which have been reported to regulate vasopressin and oxytocin secretion at the nerve endings were without effect on basal $[Ca^{2+}]_i$ or K^+-stimulated increases in $[Ca^{2+}]_i$.

INTRODUCTION

Neurotransmitter or neurohormone secretion from nerve endings relies on entry of extracellular Ca^{2+} which results from changes in membrane potential and activation of voltage-sensitive Ca^{2+} channels. Limitations imposed by the size of most vertebrate nerve endings, however, have largely precluded characterization of Ca^{2+} entry, the rise in intraterminal Ca^{2+} concentration ($[Ca^{2+}]_i$), and of the mechanisms of Ca^{2+} sequestration and efflux at single nerve endings. Therefore, much of the current understanding of these processes comes from studies of the invertebrate squid giant synapse[1,6,14,17] and radioactive tracer studies in synaptosomal preparations in vertebrate nerve endings[19,21,28,32]. Recently, development of the Ca^{2+}-sensitive fluorescent probes have allowed characterization of $[Ca^{2+}]_i$ and of depolarization induced changes in $[Ca^{2+}]_i$ in vertebrate synaptosomal[12,20,23,34] and neurosecretory endings[4]. To date, however, their use has been restricted to populations of nerve endings in a fluorometer cuvette under static conditions. There are several problems associated with this populational approach. For synaptosomal preparations homogeneity with respect to the nerve ending type is not assured nor are postsynaptic and glial elements necessarily eliminated leading to measurements which reflect an integrated response and thereby masking differences between neurotransmitter ending types. Furthermore, the static conditions do not allow for substance applica-

tion and removal nor for washout of dye effluxed from the cells during the measurements. The present study utilizes dual wavelength microspectrofluorometry of fura-2 to monitor changes in $[Ca^{2+}]_i$ in individual superfused neurosecretory nerve terminals stimulated with substances known to induce or block neurohormone release. The preparation consists entirely of nerve endings isolated from the neural lobe without contribution from post-synaptic membrane and which retain Ca^{2+}-dependent secretory responsiveness as detailed by Nordmann and coworkers[5,25].

MATERIALS AND METHODS

Isolated nerve endings were prepared from neurohypophyses of male Sprague–Dawley rats (200–250 g) using a modification of the procedure detailed by Nordmann[5,25]. The pars intermedia was removed from the neurohypophysis and the neural lobe homogenized in a buffer at 37 °C containing: 270 mM sucrose, 10 μM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mM HEPES buffered to pH 7.2 with Tris. The homogenate was then centrifuged in an Eppendorf microcentrifuge for 1.5 min. Following centrifugation the supernatant was discarded and the pellet resuspended in 0.5 ml of physiological saline at 37 °C containing: 135 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2.2 mM CaCl$_2$, 10 mM glucose buffered to pH 7.2 with 10 mM NaOH-HEPES. The endings were incubated for 30 min at 37 °C with 3 μM fura-2/AM followed by centrifugation to remove extracellular dye (0.5 min) and resuspension in the physiological saline.

Solutions of elevated $[K^+]$ were prepared by equimolar substitution with $[Na^+]$ in the physiological saline. Reductions in Na^+ alone by equimolar substitution with *N*-methyl-D-glucamine (NMG^+) showed no effect on $[Ca^{2+}]_i$. Stock solutions of nicardipine were prepared in DMSO, [D-Ala^2-D-Leu^5]enkephalin, relaxin and chole-

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cystokinin (CCK-8) in distilled water with 0.01% bovine serum albumin and veratridine in ethanol. Dilution of each substance into physiological saline was performed just prior to use. All solutions containing peptides also contained 0.05% BSA as carrier. Relaxin was a gift of the National Hormone and Pituitary Program, University of Maryland School of Medicine.

Determination of $[Ca^{2+}]_i$ in individual nerve endings was determined by dual wavelength microspectrofluorometry following loading with fura-2/AM. The microspectrofluorometry equipment and superfusion chamber utilized to monitor $[Ca^{2+}]_i$ in individual nerve endings was identical to that previously described on single cells[30,31]. All measurements were performed at 37 °C in a chamber providing rapid exchange of the superfusing physiological saline. The fluorescence ratio was converted to $[Ca^{2+}]_i$ using the equation of Grynkiewicz et al.[11]. Values of R_{min}, R_{max} and F_o/F_s were determined using an external standard calibration technique[7] resulting in values of 0.77, 19.3, and 5.5 respectively. A K_d value for fura-2 of 225 nM was taken from the literature[11]. Traces shown in the figures are representative of at least 3 separate determinations.

RESULTS

The changes in $[Ca^{2+}]_i$ in individual nerve endings were examined using elevated concentrations of K^+ in the physiological saline to induce depolarization of the membrane potential and activation of voltage-sensitive Ca^{2+} channels. The $[K^+]$ utilized (25–50 mM) has previously been shown to evoke submaximal Ca^{2+}-dependent secretion of vasopressin and oxytocin in the isolated neural lobe endings[5]. The mean basal $[Ca^{2+}]_i$ value for individual fura-2 loaded terminals in the present study was 73 ± 4 nM ($n = 126$, ± S.E.M.). Depolarization of the endings with 25 mM or 50 mM K^+ resulted in an increase in $[Ca^{2+}]_i$ of 87 ± 7 nM ($n = 24$) and 205 ± 18 nM ($n = 64$), respectively. In all cases application of elevated $[K^+]$ led to a rapid increase in $[Ca^{2+}]_i$ which was maintained for the duration of the pulse. Recovery of $[Ca^{2+}]_i$ to basal values on washout of the elevated $[K^+]$ required several minutes. Fig. 1 shows representative responses from individual terminals to pulses of 50 mM K^+. The rapid rise in intraterminal $[Ca^{2+}]$ to elevated $[K^+]$ was clearly dependent on influx of Ca^{2+} as the response was nearly completely blocked in Ca^{2+}-free media (no added Ca^{2+} plus 1 mM EGTA) and significantly reduced by 100 μM CdCl$_2$. Ca^{2+}-free medium alone reduced basal $[Ca^{2+}]_i$ values only slightly. Sustained superfusion with Ca^{2+}-free medium (20 min) and subsequent application of ionomycin (10 μM) results in a large increase in intraterminal Ca^{2+} arising from release from intraterminal stores (data not shown). Hence, Ca^{2+}-free medium does not deplete intracellular Ca^{2+}. Furthermore, no difference in the K^+-induced increase in $[Ca^{2+}]_i$ or in recovery was observed in Na^+-free medium (Fig. 1C).

Monitoring $[Ca^{2+}]_i$ in an individual nerve ending during regimes of repetitive stimulation (see Fig. 1B) allowed the effects of various Ca^{2+} channel agonists and antagonists to be quantitated with respect to a control stimulation. Furthermore, this approach allowed recovery of responsiveness to be assessed following removal of the agonist or antagonist. In particular, the increases in $[Ca^{2+}]_i$ induced by 50 mM $[K^+]$ alone, in the presence of 100 μM Cd^{2+} and following washout of Cd^{2+} were 365 ± 114, 66 ± 15, and 350 ± 107 nM ($n = 4$), respectively. The data confirm the potent inhibitory effect of Cd^{2+} as a blocker of voltage-sensitive Ca^{2+} channels.

Several types of voltage-sensitive Ca^{2+} channels are distinguishable by biophysical and pharmacological characteristics in the somata of neurons[18] and in muscle cells[2]. One of these channels termed 'L-type' characteristically shows sensitivity to dihydropyridines (DHP) while the other types lack such sensitivity[26]. Fig. 2 shows the response of individual nerve endings to the DHP, nicardipine which blocks oxytocin and vasopressin secretion[5]. Nicardipine induced a dose-dependent inhibition of the $[K^+]$ induced increase in $[Ca^{2+}]_i$ (Fig. 2C) although a slight increase could still be resolved in each terminal examined at a concentration of 25 μM nicardipine. Averaged results for the increase in $[Ca^{2+}]_i$ by two control K^+ challenges and a third elevated $[K^+]$ pulse with 5 μM nicardipine present are 166 ± 34, 173 ± 36, 51 ± 12 nM ($n = 9$), respectively. Mean resuls for an identical regime but with 25 μM nicardipine were 199 ±
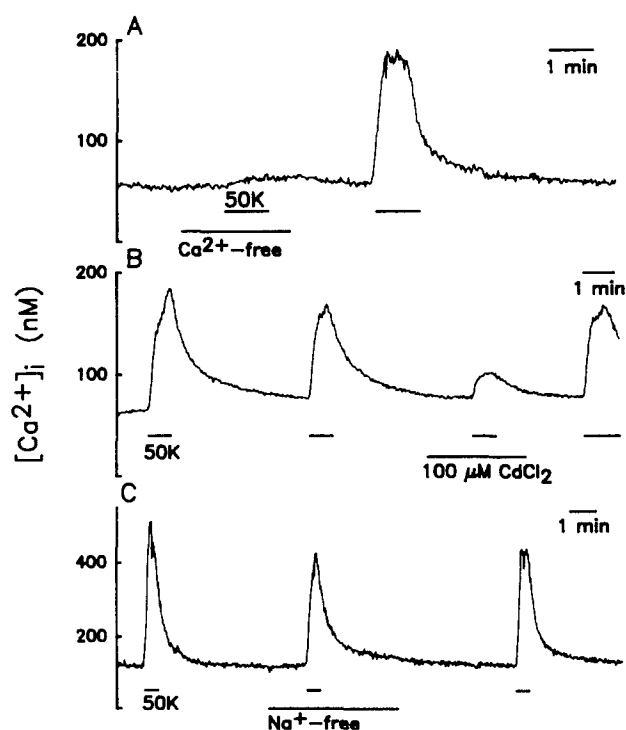

Fig. 1. Effect of K^+-induced depolarization on $[Ca^{2+}]_i$ in single nerve terminals. The K^+-induced (50 mM) rise in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+} (A) significantly reduced by Cd^{2+} (B) and unaffected by complete replacement of Na^+ by NMG^+ (C).
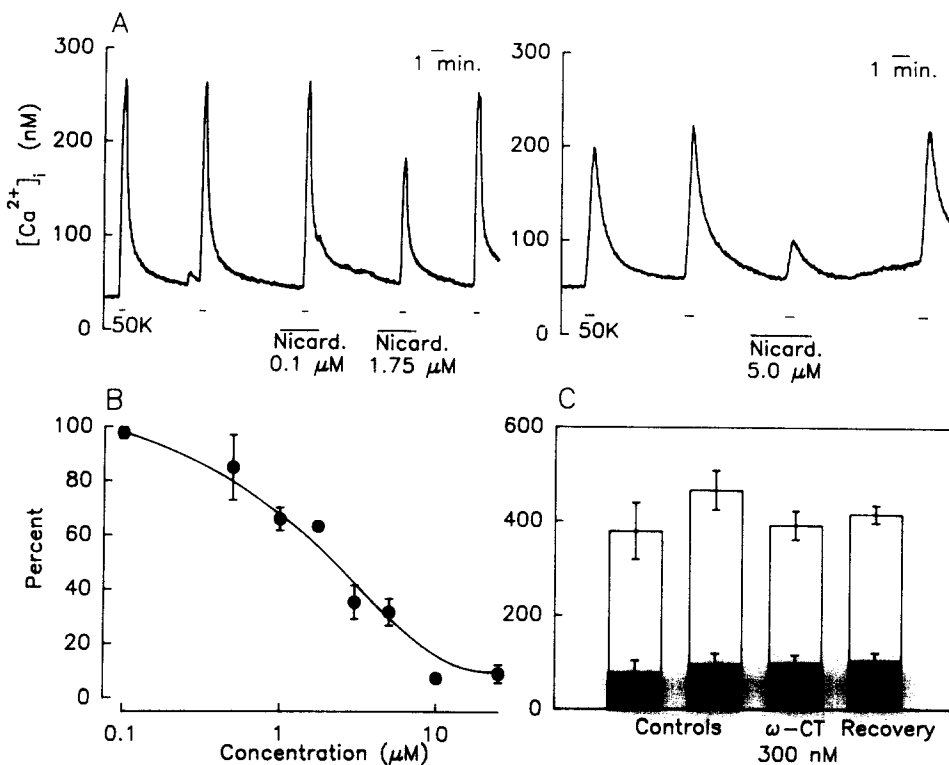
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Fig. 2. Effect of the DHP nicardipine and of ω-conotoxin GVIA on 50 mM K^+-induced increases in $[Ca^{2+}]_i$. A: addition of nicardipine prior to and during K^+-induced depolarization inhibits the evoked increase in $[Ca^{2+}]_i$. Subsequent washout of both elevated K^+ and nicardipine and restimulation with K^+ alone shows recovery of the K^+-induced increase in $[Ca^{2+}]_i$. Data shown from two separate endings. B: the effects of increasing concentrations of nicardipine on K^+-evoked $[Ca^{2+}]_i$ increase. Data for each nicardipine concentration are expressed as a percentage of the response under control conditions. Two control K^+-stimulations from which an average $[Ca^{2+}]_i$ was determined preceded each test stimulation in the presence of nicardipine. Values from at least 3 individual endings constitute each data point. C: the effect of ω-conotoxin ($n = 5$) on basal $[Ca^{2+}]_i$ (solid bars) and on a K^+-induced $[Ca^{2+}]_i$ increase (open bars).

45, 178 ± 28, 13 ± 3 nM ($n = 6$), respectively. No effect of nicardipine was observed on basal $[Ca^{2+}]_i$ values. The effects of nicardipine were fully reversible.

The peptide toxin ω-conotoxin GVIA, which blocks two distinct Ca^{2+} channel types (L and N) in chick sensory neurons[10] exhibits saturable binding and potently inhibits K^+-induced vasopressin release from the isolated neural lobe nerve endings[9]. The effects of ω-conotoxin were therefore tested on the K-evoked $[Ca^{2+}]_i$ increase. Application of ω-conotoxin GVIA at concentrations showing near maximal inhibition of K-induced vasopressin release (300 nM), however, showed no effect on basal $[Ca^{2+}]_i$ or on a 50 mM K^+-induced increase in $[Ca^{2+}]_i$ (Fig. 2C). In comparison, D-888 (desmethoxyverapamil) which also blocks voltage-dependent Ca^{2+} channels and neurohormone secretion[9] effectively reduced the K^+-evoked $[Ca^{2+}]_i$ increase. D-888 at a concentration (25 μM) which reduces vasopressin secretion by greater than 85% reduced the K-induced increase in $[Ca^{2+}]_i$ by 87% ($n = 4$) without itself affecting basal $[Ca^{2+}]_i$. The inhibition by D-888 was completely reversible.

An alternate approach to depolarize nerve endings and thereby induce Ca^{2+} entry and secretion is to utilize veratridine which maintains voltage-dependent Na^+
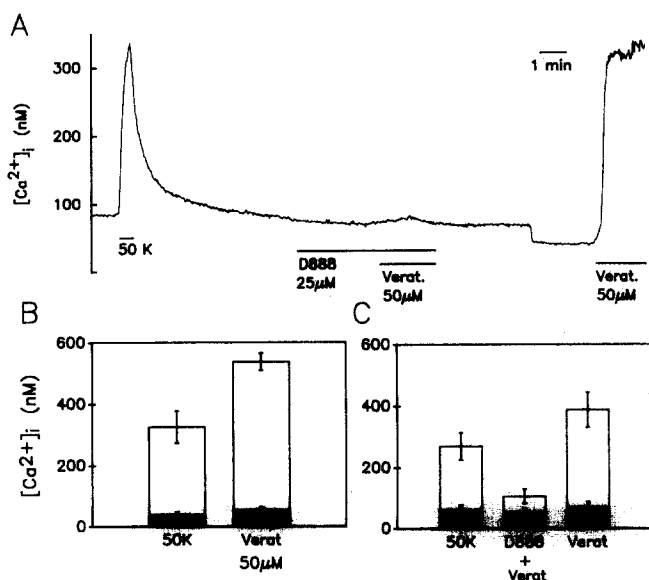

Fig. 3. Effect of veratridine on $[Ca^{2+}]_i$. A: representative recording of veratridine (50 μM) induced $[Ca^{2+}]_i$ increase and block of the effect by pretreatment with 25 μM D-888. B: averaged results ($n = 4$) comparing increase in $[Ca^{2+}]_i$ resulting from 50 mM K^+ (0.5 min) stimulation with that induced by 50 μM veratridine. C: averaged results of D-888 block of the veratridine response and comparison to a subsequent application of veratridine alone ($n = 4$). Solid and open bars in B and C represent basal and stimulated values, respectively.
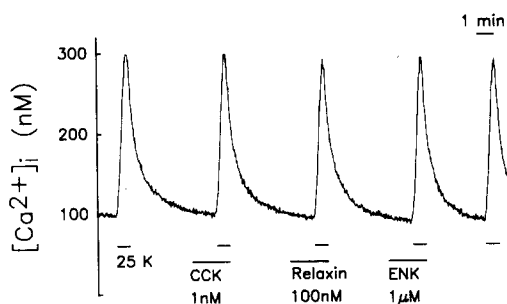
Fig. 4. Effects on $[Ca^{2+}]_i$ of relaxin, CCK and enkephalin. Representative recording of 25 mM K^+-induced increases in $[Ca^{2+}]_i$ from an individual terminal showing the lack of effect on basal or stimulated $[Ca^{2+}]_i$ values.

channels in an activated state[4,25,27]. Fig. 3 shows a representative example of the effects of veratridine on an individual nerve ending. Veratridine produced a rapid maintained increase in $[Ca^{2+}]_i$ which was generally greater in amplitude than that induced by a 0.5-min pulse of 50 mM $[K^+]$ (Fig. 3B). Prior application of D-888 effectively blocked the entry of Ca^{2+} arising from the depolarization induced by veratridine (Fig. 3A,C). Nicardipine (25 μM) added prior and during veratridine application was also effective at reducing the depolarization-induced increase in $[Ca^{2+}]_i$.

A number of peptides have been suggested to exert a regulatory influence on $[Ca^{2+}]_i$ in the neural lobe nerve endings based on their effects to potentiate or reduce oxytocin or vasopressin secretion. The effects of 3 of these compounds at doses which exert maximal effects on secretion are shown in Fig. 4. Relaxin, which reduces basal but potentiates both electrical and K^+-evoked secretion[8] showed no effect either on basal $[Ca^{2+}]_i$ or a 25 mM K^+ ($n = 4$) or 50 mM K^+ ($n = 4$) induced increase in $[Ca^{2+}]_i$. Cholecystokinin (CCK) has recently been suggested to induce mobilization of Ca^{2+} from intraterminal stores and thereby evoke secretion even in the absence of electrical or K-induced stimulation[3]. However, no effect of CCK at 1 or 10 nM on basal $[Ca^{2+}]_i$ or on 25 mM K^+-stimulated increase in $[Ca^{2+}]_i$ ($n = 4$) was observed. Prolonged application of CCK (1 nM, 25 min), also showed no effects on $[Ca^{2+}]_i$ (data not shown). No effect of enkephalin, which has also been suggested in some studies to regulate secretion from these endings (see ref. 24 for review), on either basal $[Ca^{2+}]_i$ or on 25 mM K^+-stimulated increase in $[Ca^{2+}]_i$ ($n = 3$) was observed.

DISCUSSION

The present study demonstrates the applicability of quantitatively characterizing in individual nerve terminals, loaded with the Ca^{2+}-sensitive fluorescent indicator fura-2, changes in $[Ca^{2+}]_i$ resulting from stimulation. This

approach overcomes the masking of differences between nerve ending types and of contributions from non-presynaptic terminal membrane as occurs when properties of Ca^{2+} influx or rise in intraterminal $[Ca^{2+}]$ are characterized on a population of terminals. Comparison of the results from the present study with those from fura-2 measurements on a population of neural lobe endings[4] show differences which likely result from the increased sensitivity of the individual terminal approach. These include a much lower value for basal $[Ca^{2+}]_i$ (i.e. 73 nM) than previously reported (350–420 nM) and the lack of a substantial reduction in $[Ca^{2+}]_i$ by removing extracellular Ca^{2+}. In addition, nicardipine which produced a complete block of the K^+-stimulated increase in $[Ca^{2+}]_i$ in the populational study produced a significant but incomplete block at the single terminal level. Although the modest increases in $[Ca^{2+}]_i$ to K^+-induced depolarization could be representative of heavy loading of fura-2 several observations suggest this not to be the case. These include: (1) a rapid rate of rise in $[Ca^{2+}]_i$ on addition of elevated $[K^+]$, (2) a rapid and complete recovery of $[Ca^{2+}]_i$ following stimulation; (3) a similar increase in $[Ca^{2+}]_i$ to repetitive pulses of elevated $[K^+]$ and (4) a moderate absolute fluorescent intensity and an estimate of basal $[Ca^{2+}]_i$ similar to that reported for most excitable and non-excitable cells.

The nerve endings of the neural lobe represent only two classes of endings: those arising from vasopressin or oxytocin neurons. Although differences exist in the patterning of action potentials invading oxytocin and vasopressin terminals during secretory demand they exhibit similarity in their secretory time course and in their susceptibility to block by various pharmacologic compounds when exposed to similar stimulus regimes[5,25]. This is not the case for all synaptosomal or mixed terminal preparations[29]. In the present study basal $[Ca^{2+}]_i$, $[K^+]$-stimulated changes in $[Ca^{2+}]_i$ and susceptibility to block of Ca^{2+} influx by the DHP nicardipine were similar in each of the terminals examined. Thus, vasopressin- and oxytocin-containing terminals are similar in that the K^+-induced rise in $[Ca^{2+}]_i$ is largely mediated by the DHP sensitive, L-type voltage-sensitive Ca^{2+} channels. This conclusion is supported by the recent description using patch-clamp methodology of two Ca^{2+} channel types present on these terminals which exhibit characteristics similar to L and N channel types[13]. The lack of a significant contribution by the N type channel to the rise in $[Ca^{2+}]_i$ by a 0.5-min elevated $[K^+]$ pulse is supported by the rapid rate of decay (10–20 ms) of Ca^{2+} current carried through these channels at membrane potentials greater than –10 mV. These resuls differ from those of some synaptosomal preparations where DHPs have little effect on K^+-induced $^{45}Ca^{2+}$ influx or trans-

mitter secretion[28,32] although in some cases sensitivity to DHPs has been observed[33]. Furthermore, synaptosomal preparations generally show a fast phase of calcium entry that terminates within seconds which is attributed to voltage-sensitive Ca^{2+} channels that rapidly inactivate[19]. The maintenance of the K^+-induced increase in $[Ca^{2+}]_i$ for the duration of the K^+ pulse, in the present study, suggests that in these neurosecretory terminals depolarization induced Ca^{2+} influx is maintained. The likelihood of Na^+/Ca^{2+} exchange to maintain the Ca^{2+} influx under depolarizing conditions as suggested for some synaptosomal preparations[22] is unlikely based on the observation of a similar K^+-induced rise in $[Ca^{2+}]_i$ in Na^+-free medium. Clearly the contribution of various voltage-sensitive Ca^{2+} channel types and of their influence on secretion may vary with the nerve ending type.

Relaxin, CCK and leucine enkephalin have been suggested to exert regulatory influences on vasopressin and oxytocin secretion at the level of the nerve endings by affecting $[Ca^{2+}]_i$[3,8,24]. However, in the present study, none of these peptides were found to affect either basal $[Ca^{2+}]_i$ or K^+-stimulated increases in $[Ca^{2+}]_i$. These results are especially interesting for CCK which is co-localized[16] and released[15] with oxytocin and whose effects have been suggested to play a local rather than hormonal role based on a molar concentration of CCK in the rat neural lobe several orders of magnitude less than oxytocin. Additionally, a recent report showed CCK to potently induce secretion of both oxytocin and vasopressin in the absence of either electrical or K^+-induced depolarization and independent of medium Ca^{2+} thus suggesting its actions may be mediated by mobilization of intracellularly stored Ca^{2+} (ref. 3). The results of the present study would suggest either that CCK exerts its stimulatory effects via a messenger pathway other than Ca^{2+} or its effects occur secondarily resulting from actions on glia or pituicytes. These latter cell types are largely missing in the isolated terminal preparation. Oxidation and hence reduction of biologic activity of the CCK peptide cannot explain the lack of effect as utilizing identical methodology results, at 1 nM CCK, in near maximal mobilization of intracellular calcium in isolated pancreatic acini[31]. The effects of fura-2 loading alone on the ability of the above peptides to modulate secretion has not been tested although heavier fura-2 loading of the isolated terminals has previously found no reduction in elevated $[K^+]$ induced vasopressin secretion[4]. While these results do not rule out a regulatory influence of any of these peptides they strongly suggest their actions do not include direct effects on Ca^{2+} influx mediated via DHP-sensitive Ca^{2+} channels, or mobilization of Ca^{2+} from intraterminal stores. While the effects of enkephalin on release have been primarily studied on intact isolated neural lobes the effects of both relaxin and ω-conotoxin have been documented on the isolated nerve endings. The kinetic limitations of this study preclude conclusions related to the possible modulatory effects of enkephalin or relaxin or of blocking effects of ω-conotoxin on Ca^{2+} influx via N-type Ca^{2+} channels. It is also possible that the modulatory effects of enkephalin and relaxin occur independently of $[Ca^{2+}]_i$ regulation. The lack of effect of ω-conotoxin on the K^+-evoked rise in $[Ca^{2+}]_i$ in this study, but effective block by the L-type DHP Ca^{2+} channel blocker nicardipine, suggests there may be differential susceptibility to block by ω-conotoxin among DHP-sensitive Ca^{2+} channels in excitable cell types.

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