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THE CONTROL OF INTRACELLULAR CALCIUM AND NEUROTRANSMITTER RELEASE IN GUINEA PIG-DERIVED CEREBRAL CORTICAL SYNAPTONEUROSOMES

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Abstract—Synaptoneurosomes are a simply derived brain vesicular preparation which are believed to contain elements of both presynaptic and postsynaptic material. Inositol phosphates production and neurotransmitter release in the synaptoneurosome have previously been shown to be under the control of a number of receptor agonists. However, there have been few investigations of the role of intracellular calcium ($[Ca^{2+}]_i$) in these events. In this study we report that potassium (K^+; 50 mM) was able to increase $[Ca^{2+}]_i$ and subsequently release [^{3}H]noradrenaline in guinea pig cerebral cortical synaptoneurosomes via activation of dihydropyridine-insensitive, voltage-sensitive calcium channels. Veratridine (30 μM) produced similar effects but these involved activation of sodium channels which could be blocked by pre-incubation with tetrodotoxin (0.15 μM). A number of agonists were used to investigate possible modulation of these events and to look for agonist-stimulated mobilization of $[Ca^{2+}]_i$. No evidence was found for either receptor-mediated release of calcium from intracellular stores or for modulation of K^+-induced neurotransmitter release. This might be related to the observed passive entry of calcium through the synaptoneurosomal membrane and the subsequently high levels of $[Ca^{2+}]_i$.

Transformed cell lines of neuronal origin have been used to provide useful information relating to biochemical events associated with the CNS. However, there are doubts with regard to their validity as models of directly derived CNS tissue. Synaptosomes have long been used to study nerve terminal activity as they are simple to prepare and retain many of the functional and morphological properties of intact neuronal tissue [1, 2]. They are believed to consist of only presynaptic elements and have been widely used, for instance, in studies of neurotransmitter release and receptor-mediated signal transduction [3–6]. However, many receptor-mediated events only occur at postsynaptic sites, thereby limiting the usefulness of this preparation.

On the other hand, synaptoneurosomes are a simply derived brain vesicular preparation believed to contain *both* presynaptic and postsynaptic elements [7]. Microscopic examination has revealed that this preparation contains an abundance of "snowman-shaped" entities which can be purified by filtration. Electron microscopic investigation has shown these particles to consist of synaptosomes with attached and resealed postsynaptic entities [8].

This preparation has been used to investigate inositol phospholipid turnover [9–11] and release of neurotransmitter [12] but no studies have looked directly at the mechanisms controlling intracellular calcium ($[Ca^{2+}]_i$‡) and the release of neurotransmitters.

In this study, a number of agonists were investigated for their effects on $[Ca^{2+}]_i$ in guinea pig cerebral cortical synaptoneurosomes. Although agonist-mediated phosphoinositide hydrolysis has been reported to occur in synaptosomes [9–11], we present evidence here which suggests that this does not lead to subsequent mobilization of $[Ca^{2+}]_i$ from intracellular stores, but that increases in $[Ca^{2+}]_i$ can occur via influx. Furthermore, the same conditions which inhibit this calcium entry phenomenon have similar effects on the release of [^{3}H]noradrenaline.

MATERIALS AND METHODS

Preparation of synaptoneurosomes. Dunkin–Hartley guinea pigs (approx. weight 300–400 g) were used in all of the experiments. Synaptoneurosomes were prepared according to the method of Gusovsky and Daly [9]. Briefly, following cervical dislocation the brain was quickly removed and the cortex dissected out on ice. The tissue was homogenized by hand using a tightly fitting glass–teflon homogenizer containing 10 mL of ice-cold Krebs–Henseleit–bicarbonate buffer (pH 7.4) pre-equilibrated with a mixture of 95% O_2–5% CO_2. The resulting homogenate was centrifuged at 1000 *g* for 15 min at 4°.

Calcium measurements. The pellet was resuspended in 10 mL of a simple saline buffer ($CaCl_2$,

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‡ Abbreviations: $InsP_x$, inositol phosphates; $[Ca^{2+}]_i$, intracellular calcium; TTX, tetrodotoxin; VSCCs, voltage-sensitive calcium channels; $InsP_3$, inositol 1,4,5-trisphosphate; 5-HT, 5-hydroxytryptamine.

2 mM; NaCl, 145 mM; glucose, 10 mM; KCl, 5 mM; $MgSO_4$, 1 mM and HEPES, 10 mM; pH 7.4) followed by incubation with fura-2 acetoxy methyl ester (5 μM) at 37° for 10 min. At the end of this loading period, excess dye was removed by centrifugation, the synaptoneurosomes resuspended in fresh buffer, and left at room temperature until use. Each calcium time course was preceded by a rapid spin in a microcentrifuge followed by resuspension in fresh buffer.

All experiments were carried out using a Perkin–Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm (approx. every 1.6 sec), recording at 500 nm. The time course for each calcium measurement was 200 sec with drugs added in 10-μL aliquots at the times indicated.

Manganese entry. In some experiments recordings were made at an excitation wavelength of 360 nm (the "isobestic point"). Recordings at this wavelength are independent of calcium and depend only on the concentration of the dye itself. Manganese (Mn^{2+}) quenches fura-2 fluorescence at all wavelengths and under resting conditions is largely excluded from the cytoplasm. Following agonist stimulation it is believed to enter via a similar pathway to calcium ions. Thus, exciting at 360 nm, in the presence of extracellular Mn^{2+}, can be used to investigate entry of calcium into the cytoplasm [13–16]. Mn^{2+} (200 μM) was added at the beginning of the time course, in the absence of extracellular Ca^{2+} ions. Recordings of individual wavelengths were made over a time course of 200 sec.

Calibration. At the end of each time course, ionomycin (20 μM) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and R_{min}. Autofluorescence was determined using a separate cuvette following the addition of Mn^{2+} (5 mM) after the ionophore, ionomycin (20 μM). Using these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* [17].

Phosphoinositide hydrolysis. Guinea pig cerebral cortical slices were prepared and prelabelled with [^{3}H]*myo*-inositol prior to the preparation of synaptoneurosomes as described by Hollingsworth and Daly [18]. Phosphoinositide hydrolysis was assessed by measuring the accumulation of total [^{3}H]inositol phosphates ([^{3}H]$InsP_x$) in the presence of 5 mM lithium chloride following a 45 min period of stimulation with carbachol and K^+.

Neurotransmitter release. This was carried out using the method of Ebstein *et al.* [12]. Briefly, synaptoneurosomes were prepared as above and the final volume adjusted to 10 mL (in Krebs–Henseleit). The suspension was incubated with 0.2 μM [^{3}H]-noradrenaline for 30 min at 37° in a shaking waterbath. After the uptake period the synaptoneurosomes were centrifuged (1000 *g* for 5 min) and resuspended in fresh buffer three times (to remove excess radiolabel). The final volume was adjusted to 100 mL.

Aliquots of the suspension (0.5 mL) were carefully layered on small plastic columns containing 1 mL of Sephadex G-10 resin (100 g Sephadex G-10 in 700 mL water). Initially five 2-mL fractions of Krebs–Henseleit buffer (with or without calcium; 2 mM)

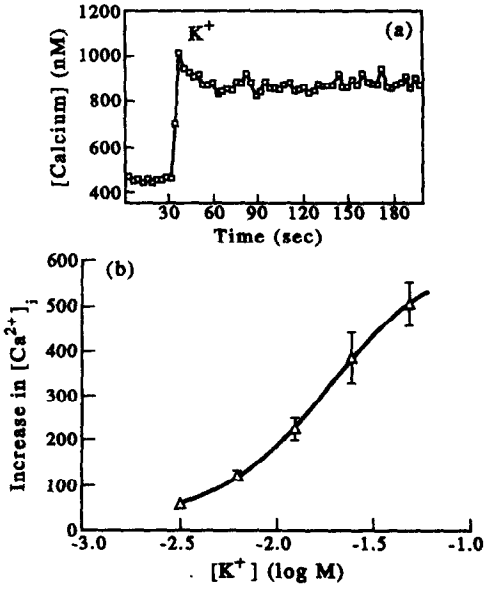

Fig. 1. (a) The effect of potassium (K^+; 50 mM) on $[Ca^{2+}]_i$ in fura-2-loaded synaptoneurosomes. Fluorescence values were measured following excitation at 340 and 380 nm and the ratio used to determine $[Ca^{2+}]_i$. The graph is typical of two others. (b) The concentration–effect relationship for potassium. Data are the means of three separate determinations; vertical error bars represent SEM.

were washed through the columns by gravity flow, at room temperature, to investigate the profile of release. These were followed by five 2-mL fractions containing K^+ (50 mM; again with or without calcium). The fractions were collected in scintillation vials and 10 mL of Picofluor scintillation fluid added. Using the resulting information a standard protocol was observed: after loading onto the columns the synaptoneurosomes were washed with 10 mL of buffer (containing calcium; 2 mM). The initial wash was discarded and a second portion of buffer (2 mL) was applied (with or without drug) and collected. This was followed by buffer (2 mL) containing K^+ or veratridine. The amount of released tritium due to depolarization was calculated by obtaining the difference between the first and second washes. All significance testing was carried out using this calculated difference but for clarity data represented has been expressed as a percentage increase above basal. The effect of each drug alone was assessed by comparing release in the presence of drug and absence of K^+, with that in the absence of drug and K^+.

Ebstein *et al.* [12] in the same preparation demonstrated, using chromatographic analysis, that 70–80% of the tritium released following stimulation with K^+ represented [^{3}H]noradrenaline.

Materials. Sephadex G-10 resin, veratridine, angiotensin-II, carbachol, 5-hydroxytryptamine (5-HT), clonidine, nickel, glutamate, tetrodotoxin (TTX), EGTA and isradipine were supplied by the Sigma Chemical Co. (Poole, U.K.), with fura-2 AM and ionomycin from Calbiochem (Novobiochem,

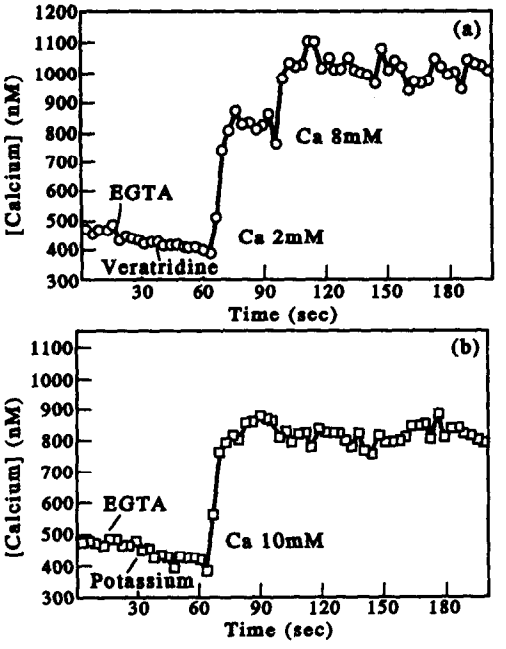

Fig. 2. The effect of EGTA on the calcium response to potassium (b; 50 mM) and veratridine (a; 30 μM). The synaptoneurosomes were suspended in nominally calcium-free medium. Addition of EGTA (1.25 mM) inhibited the responses to both agents. However, when sufficient calcium (Ca; 10 mM) was added to saturate the chelating agent, both responses returned to control values. The graph shown is typical of two others.

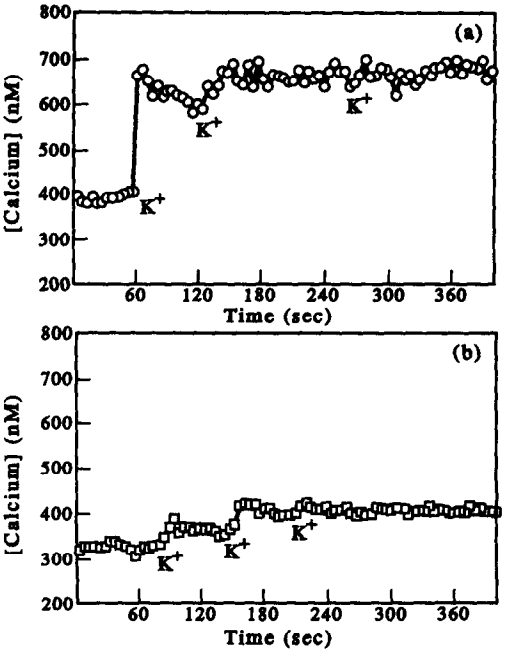

Fig. 3. The effect of the inorganic calcium channel blocker, nickel (1 mM). In (a), potassium (K^+; 25 mM) significantly increased $[Ca^{2+}]_i$. Subsequent additions had very little effect. When nickel was included in the extracellular medium (b), the response to potassium was significantly attenuated. The graph shown is typical of two others.

Nottingham, U.K.). 2-Chloradenosine was supplied by Boehringer Mannheim (Lewes, U.K.) with [^{3}H]-noradrenaline from Amersham (Amersham, U.K.). Columns were obtained from BioRad (Watford, U.K.). [^{3}H]*myo*-Inositol was obtained from NEN (Stevenage, U.K.).

Statistics and data analysis. Calcium data was captured using the ICBC program supplied by Perkin–Elmer and imported to the graphics program Sigma-Plot (Jandel).

Significance testing was carried out using a Student's *t*-test on non-transformed data.

RESULTS

Effect of K^+ *on* $[Ca^{2+}]_i$

Addition of K^+ (50 mM) produced a rapid increase in $[Ca^{2+}]_i$ of 499 ± 34 nM (N = 4) from a resting value of 408 ± 32 nM (N = 4) which was maintained above basal for at least 3 min (Fig. 1a). The response was also found to be concentration-dependent (Fig. 1b). Omission of calcium from the extracellular medium caused a significant reduction in the size of the response (69 ± 3% of control; N = 3, $P < 0.05$). Addition of EGTA (1.25 mM) to nominally calcium-free medium prevented the K^+-stimulated increase in $[Ca^{2+}]_i$ completely (Fig. 2b); however, addition of calcium (10 mM) caused a similar increase to that observed in the absence of the chelating agent. Addition of the inorganic channel blocker, nickel (1 mM), similarly inhibited K^+-stimulated calcium rises (Fig. 3), but the dihydropyridine, isradipine (3 μM), failed to have any effect (99 ± 1%; N = 3).

The synaptoneurosomes were also challenged with agonists which have previously been associated with $InsP_x$ production [9–11]. However, histamine (300 μM) and carbachol (100 μM) failed to have any effect on $[Ca^{2+}]_i$ (N = 3; data not shown).

Mn^{2+} *influx*

Mn^{2+} (200 μM) was added to the incubation medium in order to investigate the possibility of K^+-stimulated entry (Fig. 4a). When recordings were made at excitation wavelengths of 340 and 360 nm, there was a maintained drop in both signals immediately after addition of Mn^{2+} (due to quenching of extracellular fura-2). Addition of K^+ (25 mM), in the absence of extracellular calcium, appeared to have a small effect at both wavelengths (Fig. 4a).

Effect of veratridine on $[Ca^{2+}]_i$

Addition of the sodium channel activator, veratridine, resulted in a concentration-dependent, sustained increase in $[Ca^{2+}]_i$ (Fig. 5). The response showed a number of characteristics similar to those exhibited following addition of K^+ i.e. sensitivity to calcium (omission of extracellular calcium reduced responses to 42 ± 2% of controls; N = 3 and inclusion of EGTA prevented the response completely; Fig. 2a) and the inorganic calcium channel blocker, nickel

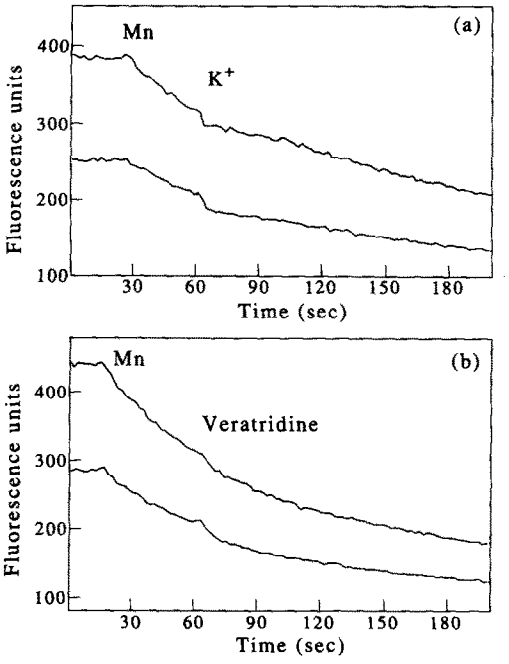

Fig. 4. Single wavelength recordings in fura-2-loaded synaptoneurosomes suspended in nominally calcium-free medium (see text), stimulated with potassium (K^+; a; 50 mM) and veratridine (b; 30 μM). The emission wavelength was set at 500 nm with excitation at 340 (a) and 360 (b) nm. Addition of manganese (Mn^{2+}; 200 μM) resulted in decreased fluorescence at both wavelengths. Both potassium and veratridine slightly increased the rate of manganese entry (indicated by the change in the 360 nm trace). This graph is typical of two other experiments.

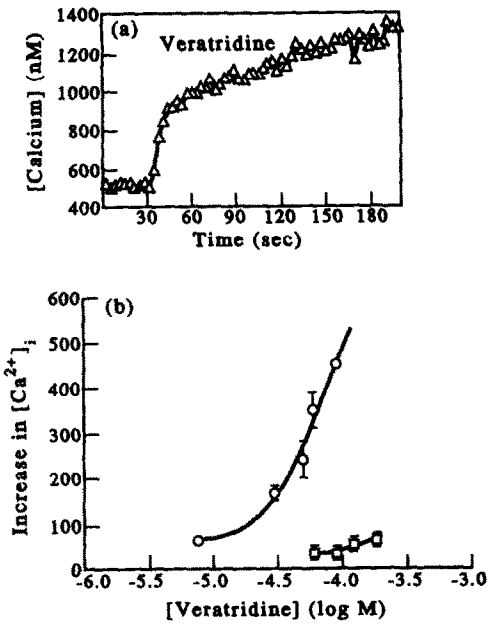

Fig. 5. The upper panel shows the effect of veratridine (30 μM) on $[Ca^{2+}]_i$ in fura-2-loaded synaptoneurosomes. Fluorescence values were measured following excitation at 340 and 380 nm and the ratio used to determine $[Ca^{2+}]_i$. The graph is typical of two others. The lower panel shows the concentration–effect relationship for veratridine in the presence (□) and absence (○) of TTX (0.15 μM). Data are the means of three separate determinations; vertical error bars represent SEM.

(data not shown). In addition, pre-incubation with the sodium channel blocker, TTX (0.15 μM), antagonized its effects (Fig. 5b; the K^+-stimulated increase in $[Ca^{2+}]_i$ (12.5 mM) was unaffected by TTX; 95 ± 9% of control values; N = 3).

Modulation of the K^+-mediated response by receptor stimulation

Synaptoneurosomes were pre-incubated for 5 min with a number of agonists and their effects on sub-maximal K^+ stimulation (12.5 mM) assessed. Clonidine (10 μM), 5-HT (0.5 mM) and 2-chloroadenosine (0.9 μM) failed to show any significant effects (105 ± 6%, 105 ± 3% and 94 ± 11% of control responses, respectively).

Phosphoinositide hydrolysis

Stimulation of synaptoneurosomes with the cholinergic agonist, carbachol (1 mM), increased the accumulation of $[^3H]InsP_x$ to 203 ± 26% of basal (N = 4; $P < 0.05$). Similarly, elevating the concentration of K^+ in the medium to 31 mM increased $[^3H]InsP_x$ accumulation to 172 ± 34% of basal (N = 3; $P < 0.05$).

Release of [^{3}H]noradrenaline

K^+ (50 mM) stimulated the release of [^{3}H]noradrenaline from pre-loaded synaptoneurosomes (Fig. 6a). The response was again concentration-dependent (Fig. 6c) and was significantly attenuated when calcium was omitted from the extracellular medium (Fig. 6b). Nickel (1 mM), but not the dihydropyridine calcium channel blocker, isradipine (3 μM), also reduced the K^+-dependent release of [^{3}H]noradrenaline (Fig. 7a). Veratridine (30 μM) was also able to stimulate the release of neurotransmitter and was sensitive to pre-treatment with TTX (0.15 μM; Fig. 7b).

Modulation of release

Some modulation of the K^+-stimulated release mechanism was achieved by pre-incubation with clonidine (10 μM). This α_2-adreneoceptor agonist significantly reduced K^+-stimulated release to 81 ± 5% of control ($P < 0.05$; N = 3). However, a similarly sized reduction (84 ± 7%, $P < 0.05$; N = 3) was observed in synaptoneurosomes challenged with clonidine alone.

DISCUSSION

K^+ and veratridine significantly increased $[Ca^{2+}]_i$ in guinea pig-derived cortical synaptoneurosomes. Both responses were dependent on extracellular calcium and were significantly inhibited by the inorganic calcium channel blocker, nickel. The increase in $[Ca^{2+}]_i$ was found to be necessary for neurotransmitter release. However, despite the

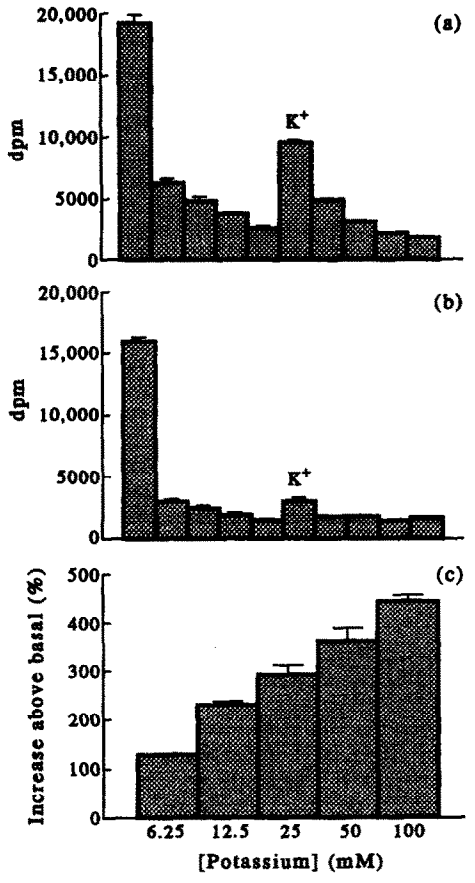

Fig. 6. The release profiles from synaptoneurosomes prelabelled with [^{3}H]noradrenaline (see text) in the presence (a; 2 mM) and absence (b) of extracellular calcium. The synaptoneurosomes were added to plastic columns containing Sephadex G-10 resin and fractions of Krebs–Henseleit buffer (2 mL each fraction) eluted and collected under gravity flow. After five fractions had been collected, buffer containing potassium (K^+; 50 mM) was passed through the columns and a further 10 mL was collected. Potassium increased the release of [^{3}H]noradrenaline but only in the presence of extracellular calcium. Panel (c) represents the concentration–response relationship for potassium-stimulated neurotransmitter release. Initially 10 mL of buffer (calcium; 2 mM) was eluted through the columns and discarded. This was followed by a 2 mL portion of calcium buffer, which was collected, followed by a second 2 mL portion containing various concentrations of potassium. The amount of release was calculated by determining the difference between these two fractions. The data shown are the mean of four determinations (error bars represent SEM) and have been normalized to show the percentage increase over basal.

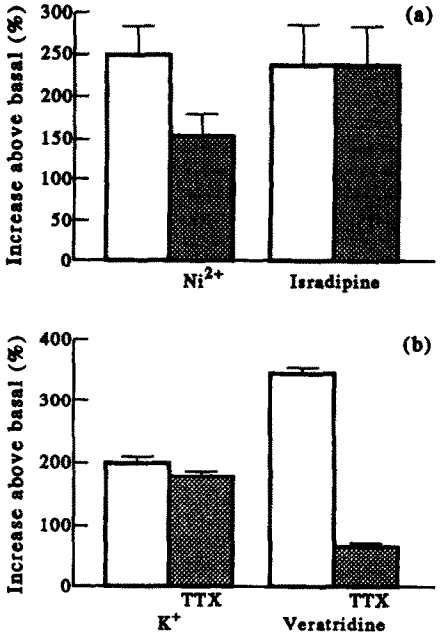

Fig. 7. Panel (a) shows the effects of nickel (Ni^{2+}; 1 mM) and isradipine (3 μM) on potassium-stimulated (50 mM) release. Nickel significantly inhibited ($P < 0.05$; Student's *t*-test carried out on non-transformed data) the response, but isradipine had no effect. Data are the mean of three experiments each of four determinations; error bars represent SEM. Panel (b) shows the effect of TTX (0.5 μM) on potassium- (K^+; 50 mM) and veratridine- (30 μM) induced release. TTX significantly ($P < 0.001$) inhibited the effects of veratridine. Data are the mean of four determinations; error bars represent the SEM.

agonist-stimulated accumulation of $InsP_x$ measured in this preparation (a result consistent with previous reports [9–11]) there was no agonist-stimulated mobilization of calcium from intracellular stores.

Addition of K^+ produced a maintained, concentration-dependent increase in $[Ca^{2+}]_i$ which required extracellular calcium and was inhibited by the inorganic calcium channel blocker, nickel, but was unaffected by the dihydropyridine, isradipine. This evidence suggests that depolarization of the plasma membrane leads to activation of voltage-sensitive, dihydropyridine-insensitive calcium channels. Furthermore, the lack of effect of TTX on this response indicates that sodium channels do not contribute to the calcium response. This is not surprising since it has previously been shown in synaptosomes that addition of K^+ leads to a depolarization of the plasma membrane (approximately 30 mV) with negligible sodium influx [19, 20]. This condition induces a fast activation of voltage-sensitive calcium channels (VSSCs) which subsequently inactivate [21, 22]. As a result, the observed maintained calcium plateau is perhaps not what would be expected, and a return to pre-stimulus levels might have been predicted assuming that the increased amount of $[Ca^{2+}]_i$ can be sequestered. Indeed, it is possible that synaptoneurosomes lack the necessary calcium buffering capacity needed to reverse the calcium increase. However, this is unlikely as addition of TTX *after* veratridine resulted in a partial reversal of the calcium response (data not shown), indicating some ability of the preparation to sequester calcium. A more likely explanation for the maintained calcium elevation is residual conductance through largely inactivated calcium

channels as was suggested by Tibbs *et al.* [23] for synaptosomes.

Addition of the sodium channel activator, veratridine, produced an increase in $[Ca^{2+}]_i$ which was sensitive to the omission of extracellular calcium, the inclusion of nickel, and which was abolished by pre-treatment with TTX. There were some differences in the time course of the veratridine response compared with that elicited by K^+. The K^+ response reached a maximum within 10 sec and then declined slightly to a plateau which was maintained for at least 3 min. The response to veratridine, on the other hand, after a rapid initial phase increased slowly for the remainder of the experiment. The veratridine response, therefore, might involve additional mechanisms. The initial increase in intracellular sodium could lead to a reversal of the Na^+/Ca^{2+} exchanger, known to be present in the plasma membrane [24], leading to further calcium entry. Furthermore, it is possible that some calcium entry might take place directly via sodium channels.

It has been shown previously [9–11] that a number of agonists, including carbachol and histamine, are able to stimulate the production of $InsP_x$ in guinea pig-derived cerebral cortical synaptoneurosomes, and inositol-1,4,5-trisphosphate ($InsP_3$) has been linked with mobilization of calcium from intracellular stores in a large number of cellular preparations [25–28]. However, our experiments revealed agonist-stimulated phosphoinositide hydrolysis but failed to show any evidence for mobilization of $[Ca^{2+}]_i$ in synaptoneurosomes, with histamine and carbachol having no significant effect on $[Ca^{2+}]_i$.

Addition of EGTA abolished the calcium responses elicited by K^+ and veratridine, and addition of sufficient calcium to saturate the chelating agent caused a similar increase in $[Ca^{2+}]_i$ to that observed in the absence of EGTA. The results need to be viewed with some caution however, as EGTA has been shown to deplete calcium in intracellular storage sites [29], although this effect generally requires longer pre-incubation. Thus, although some contribution from intracellular stores cannot be entirely ruled out it would appear that increases in $[Ca^{2+}]_i$ are predominantly due to calcium influx. The results therefore suggest either the absence of intracellular stores in this preparation or perhaps a lack of the "calcium mobilizing machinery". For instance, there is no direct evidence showing the presence of receptors for the calcium mobilizing secondary messenger $InsP_3$ in synaptoneurosomes, and calcium mobilization could be prevented by the absence of $InsP_3$ receptors or by them being desensitized. In this regard, it is interesting to note that the basal levels of $[Ca^{2+}]_i$ in the synaptoneurosome are much higher (approximately 400 nM) than is usual in a number of cell types e.g. N1E-115 neuroblastomas where $[Ca^{2+}]_i$ is around 100 nM [28] and $InsP_3$ receptor binding has been suggested to be reduced by $[Ca^{2+}]_i$ [30]. Before any conclusions can be drawn concerning the presence (or absence) of $InsP_3$-sensitive calcium stores it would be necessary to investigate the effect of $InsP_3$ by either direct injection or application to a permeabilized preparation.

Mn^{2+} has been used in a number of cellular preparations as a marker for calcium entry [13–16] and its addition to synaptoneurosomes suspended in nominally calcium-free medium resulted in a continuous fall in both 340 and 360 nm wavelengths. These results indicate that the preparation is very permeable to Mn^{2+}. This may explain why addition of K^+ resulted in a smaller change in the 360 nm signal than might have been expected. It is also possible that there was only a small entry of Mn^{2+} observed because a proportion of the increase in $[Ca^{2+}]_i$ was due to release from caffeine/ryanodine-sensitive calcium stores triggered by a small influx of calcium through VSCCs. Although in the present study the effect of caffeine was not investigated, caffeine has been shown to release $^{45}Ca^{2+}$ from a synaptosomal preparation of rat cerebral cortex [31]. With regard to the unstimulated entry of Mn^{2+}, it is tempting to suggest that the synaptoneurosomes may exhibit a similar tendency to allow a passive entry of calcium which could explain the relatively high basal calcium values recorded in this preparation. There is considerable variability in reported values for $[Ca^{2+}]_i$ in synaptosome/synaptoneurosome preparations. Synaptoneurosomes derived from 8-day-old whole brain were found to have very high values for $[Ca^{2+}]_i$ (in excess of 2 μM) if incubated at 37° for periods greater than 30 min [32]. Indeed, it is possible that the high levels of $[Ca^{2+}]_i$ might also be responsible for the lack of any receptor-mediated modulation of the K^+ response, which might involve more subtle changes, similar to those observed in synaptosomal preparations [33, 34]. It would appear that the extracellular calcium concentration might be responsible for the high $[Ca^{2+}]_i$ level. Chandler and Crews [11] observed significant increases in both basal and K^+-stimulated $[Ca^{2+}]_i$ and $InsP_x$ production in rat synaptoneurosomes when extracellular calcium was increased between 0 and 1 mM. Furthermore, the modulations in neurotransmitter release observed by Ebstein [12] showed significant sensitivity to extracellular calcium. Thus, careful control of the extracellular calcium concentration and possible lowering of $[Ca^{2+}]_i$ by the use of calcium buffers might be necessary to reveal modulations of the responses.

An increase in $[Ca^{2+}]_i$ would appear to be an important step in the release of neurotransmitters from synaptoneurosomes. K^+ induced a significant increase in the release of [^{3}H]noradrenaline which was inhibited by the omission of extracellular calcium, by inclusion of the channel blocker, nickel, but was insensitive to the dihydropyridine, isradipine. Veratridine-induced release exhibited similar properties and was additionally sensitive to TTX. There was, however, no evidence for agonist-mediated modulation of release, just as there was no agonist-modulation of calcium entry. Clonidine appeared to have an inhibitory effect on K^+-stimulated release as reported by Ebstein *et al.* [12] but its effects on basal release were not investigated in that study and our results revealed a significant inhibition of release induced by the drug alone, making interpretation difficult.

In summary, the synaptoneurosome is a useful preparation for investigations of calcium entry via activation of VSCCs and subsequent release of

neurotransmitter. Unfortunately the lack of any receptor-control of these events limit its potential as a CNS model system.

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