Biochimica et Biophysica Acta, 1026 (1990) 126–132
Elsevier

BBAMEM 74876

Effect of maitotoxin on cytosolic Ca^{2+} levels and membrane potential in purified rat brain synaptosomes

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(Received 28 December 1989)

Key words Maitotoxin, Membrane potential, Calcium ion, cytosolic, Voltage-operated Ca^{2+} channel, Calcium ion channel, (Rat brain synaptosome)

In this study, the effects of the marine toxin maitotoxin on cytosolic Ca^{2+} levels and membrane potential in rat brain synaptosomes were evaluated. Maitotoxin (10 ng/ml) caused a remarkable increase of intrasynaptosomal Ca^{2+} levels monitored by the fluorescent probe fura-2. This increase was prevented by the removal of external Ca^{2+} ions. Tetrodotoxin, as well as the removal of extracellular Na^+ ions, failed to affect maitotoxin-induced increase of intrasynaptosomal Ca^{2+} levels. Also the complete removal of all monovalent and divalent cations, except Ca^{2+} ions, from the incubation medium (0.32 M sucrose substitution), was unable to prevent the effect of maitotoxin on intrasynaptosomal Ca^{2+} levels. Maitotoxin (0.3–10 ng/ml), produced a dose-dependent depolarization of synaptosomal membranes, which required the presence of extracellular Ca^{2+} ions. The substitution of extracellular Na^+ with choline or the removal of all cations from the incubation medium and their replacement with an isotonic concentration of sucrose (0.32 M), did not prevent the depolarizing effect exerted by maitotoxin. Also under these two ionic conditions, the effect of maitotoxin on membrane potential was critically dependent on the presence of 1 mM extracellular Ca^{2+}. The depolarizing effect exerted by maitotoxin on synaptosomal membrane potential was also observed when extracellular Ca^{2+} ions were substituted with an equimolar concentration of Ba^{2+} or Sr^{2+} ions. In summary, these results appear to suggest that, in presence of 1 mM extracellular Ca^{2+} ions, maitotoxin depolarizes synaptosomal plasmamembrane by promoting the influx of extracellular Ca^{2+} ions. This enhanced influx of Ca^{2+} causes an increase of intrasynaptosomal Ca^{2+} levels.

Introduction

Toxins acting on ionic channels have been invaluable tools for the study of these membrane proteins [1,2]

Maitotoxin (MTX) is one of the most potent marine toxin known extracted from the poisonous dinoflagellate (*Gambierdiscus toxicus* [3] This toxin has been reported to behave as a putative Ca^{2+} channel activator [4], due to its ability to stimulate $^{45}Ca^{2+}$ uptake and Ca^{2+}-dependent neurotransmitter release from PC12 cells In addition, it has been shown that MTX stimulates cardiac [5] and smooth muscle [6] contraction, hormonal release from pituitary cells [7] and neurotransmitter release from hypothalamic dopaminergic neurons [8] and cultured neuronal cells [9], all these effects of MTX have been demonstrated to be dependent on the influx of extracellular Ca^{2+} ions Therefore, the aim of this study was to investigate the ionic conditions required for the stimulation of Ca^{2+} influx exerted by MTX in purified rat brain synaptosomes For this purpose, cytosolic Ca^{2+} levels were monitored by means of the fluorescent dye fura-2 [10] Furthermore, in order to relate the transmembrane Ca^{2+} movements induced by MTX with variations of synaptosomal membrane potential, the fluorescent membrane potential-sensitive probe bis(1,3-diethylthiobarbiturate)trimethine-oxonol (bisoxonol) was used [11,12]

Abbreviations EDTA, ethylenediaminetetraacetic acid, DTT, DL-dithiothreitol, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, DMSO, dimethyl sulfoxide, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, bisoxonol, bis(1,3-diethylthiobarbiturate)trimethine-oxonol

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Materials and Methods

Synaptosomal preparation

Synaptosomal fractions from brains of adult (150–200 g body wt) male Wistar rats were prepared according to the procedure of Dunkley et al [13], using a discontinuous Percoll gradient

Briefly, rats were killed by decapitation and the brains removed and kept on ice The cerebellum and the brainstem were discarded Whole brains were homogenized using a Teflon-glass homogenizer in 9 ml/g tissue of cold sucrose medium, whose composition was (in mM) 320 sucrose, 1 EDTA, 0 25 DTT, adjusted to pH 7 4 with 1 M NaOH The homogenate was centrifuged for 10 min at $1000 \times g$ at 4° C The supernatant (S_1) was diluted to 14 ml/g of starting tissue with sucrose medium, and 2 ml of S_1 were layered on the top of a tube containing four different Percoll concentrations (from the bottom (in v/v) 23%, 15%, 10% and 3% Percoll-sucrose media), prepared using a peristaltic pump in order to achieve flat interfacies among the solutions at different Percoll concentrations The gradients were then placed in a Sorvall RCSB superspeed centrifuge and spun at 4° C for 5 min at $32000 \times g$ in a SS-34 rotor After centrifugation, the fraction at the interface between the 23% and 15% Percoll layers (fraction 4, as described by Dunkley et al [13]) which is highly enriched in viable synaptosomes [14] and which was examined in the present study by electron microscopy, was removed and diluted 5-times in a solution containing (in mM) 125 NaCl, 2 5 KCl, 1 2 KH_2PO_4, 1 2 $MgSO_4$, 5 $NaHCO_3$, 25 Hepes, 6 glucose (pH 7 4) Diluted synaptosomes were centrifuged two times at $15000 \times g$ for 15 min at 4° C, in order to remove Percoll The final pellet was resuspended in a medium containing (in mM) 145 NaCl, 5 KCl, 1 2 $MgCl_2$, 10 glucose, 10 Hepes (pH 7 4) (standard Ca^{2+}-free medium), at a protein concentration of 3–10 mg of synaptosomal proteins per ml, and immediately used for the following experimental procedures

Determination of intrasynaptosomal Ca^{2+} levels

Resuspended synaptosomes (3 mg protein/ml) were equilibrated for 10 min at 37° C in a Ca^{2+}-free medium After this period, 0 5% of bovine serum albumin (BSA) was added to the synaptosomes, to provide a carrier function which aids loading [15] 5 min after the addition of BSA, synaptosomes were exposed to 5 μM of the fluorescent Ca^{2+} indicator fura-2 acetoxymethyl ester [10] 30 min after dye loading, synaptosomes were diluted 10-fold and spun at $11000 \times g$ for 10 min, to remove the extracellular dye The synaptosomal pellet was resuspended at a protein concentration of 6 mg/ml in a Ca^{2+}-free medium and kept on ice Before each experiment, 100 μl of fura-2 loaded synaptosomes were centrifuged ($9500 \times g$ for 3 min), and the resulting pellet was resuspended in 2 ml of Ca^{2+}-free medium at 37° C and added to a thermostated quartz cuvette in a Perkin-Elmer LS-S spectrofluorimeter equipped with a magnetic ministirrer Fluorescence intensity of fura-2-loaded synaptosomes was recorded at an excitation and emission wavelength of 340 and 490 nm, respectively Calibration of the fluorescent signal was performed according to the method of Grynkiewicz et al [10], by means of the following calibration equation for a dye using intensity values at one wavelength

$$[Ca^{2+}] = K_d\left(\frac{F - F_{min}}{F_{max} - F}\right)$$

where $K_d = 225$ nM, F_{min} is the fluorescence of fura-2 after the synaptosomal lysis in presence of 2 mM external EGTA, and F_{max} is the fluorescence of fura-2 after its complete saturation with Ca^{2+} (addition of 5 mM Ca^{2+}) (see also the legend for Fig 1)

Determination of synaptosomal membrane potential variations

The lipophylic anion bisoxonol, which has been used to monitor membrane potential changes in several cell types [11,12] was added, in a concentration of 300 nM from a stock solution of 150 μM in DMSO, to the quartz cuvette of the spectrofluorimeter containing 2 ml of prewarmed medium, whose ionic composition was varied according to the experimental protocol Fluorescence intensity of the dye was recorded at an excitation and emission wavelengths of 540 and 580 nm, respectively (5 nm slits for both excitation and emission wavelengths) After 1 min from the addition of bisoxonol, 40–50 μg of synaptosomal proteins were pipetted into the cuvette Subsequent additions of MTX were made from a concentrated stock solution of 10 μg of toxin/ml in 100% methanol Bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values, since the valinomycin null-point method [12] could not be applied, due to the formation of complexes between the lipophylic anion bisoxonol and the positively charged molecule of valinomycin

Protein determination

Synaptosomal proteins were determined by the method of Bradford [16]

Materials

MTX and bisoxonol were kindly provided by Prof T Yasumoto (Sendai, Japan) and Prof T Pozzan (Padova, Italy), respectively

Results

Effect of MTX on intrasynaptosomal Ca^{2+} levels in presence or in absence of extracellular Ca^{2+} ions

Under basal conditions and in presence of 1 mM extracellular Ca^{2+}, intrasynaptosomal Ca^{2+} concentration was 271 ± 31 nM (mean ± S E , 12 determinations) This value is in close agreement with previously published results obtained in brain synaptosomes [17,18]

Subsequent addition of MTX (10ng/ml) caused a progressive rise of cytosolic Ca^{2+} levels This increase reached values close to the saturation capacity of the fluorescent dye (Fig 1A) and was completely abolished if extracellular Ca^{2+} ions were removed from the medium and the Ca^{2+}-chelator EGTA was added (Fig 1B) Interestingly, under the latter experimental condition, when Ca^{2+} was reintroduced in the incubation medium, after 2 min from the addition of MTX, a rapid increase of cytosolic Ca^{2+} levels occurred

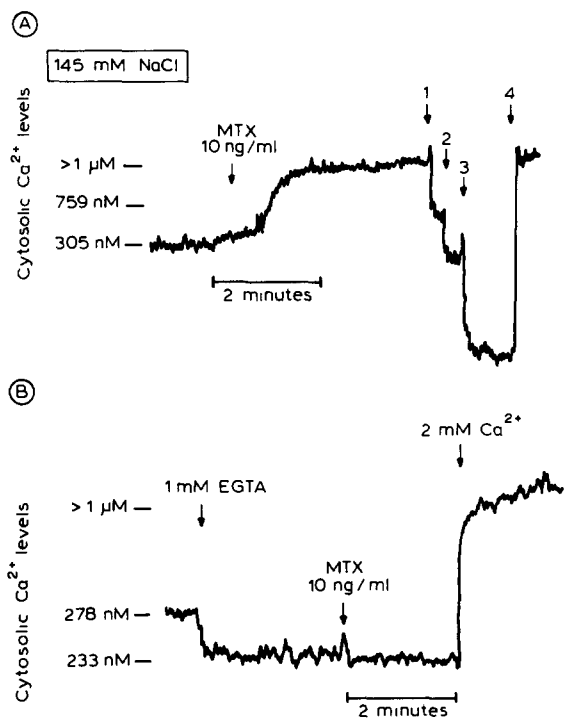

Fig 1 Extracellular Ca^{2+}-dependence of MTX-stimulated increase of intrasynaptosomal Ca^{2+} levels (A) Fura-2-loaded synaptosomes (600 μg of proteins) were resuspended in 2 ml of the standard Ca^{2+}-free medium containing 145 mM Na^+ in the spectrofluorimeter cuvette After 2 min, 1 mM Ca^{2+} was added to the synaptosomes After a period of equilibration with Ca^{2+} which lasted at least 5 min, 10 ng/ml of MTX, from a concentrated stock (10 μg/ml) were added to the synaptosomes, and fluorescence of the dye was recorded as described in Materials and Methods The right part of the trace shows the calibration of the fluorescence signal, which was achieved by subsequent addition of EGTA (2 mM, arrow 1), Tris (20 mM arrow 2), Triton X-100 (0 1%, arrow 3) and Ca^{2+} (5 mM, arrow 4) (B) after resuspension of synaptosomes in 2 ml of the standard Ca^{2+}-free medium containing 145 mM Na^+, 1 mM EGTA was added to the cuvette After about 2 min from the addition of EGTA, 10 ng/ml of MTX was added to the synaptosomes, as indicated by the arrow Two min later, Ca^{2+} ions (2 mM), a concentration saturating extracellular EGTA, were pipetted into the cuvette Calibration of fura-2 fluorescence signal was performed as in Fig 1A The experiments shown in this and in the following figures have been performed at least three times, always giving comparable results

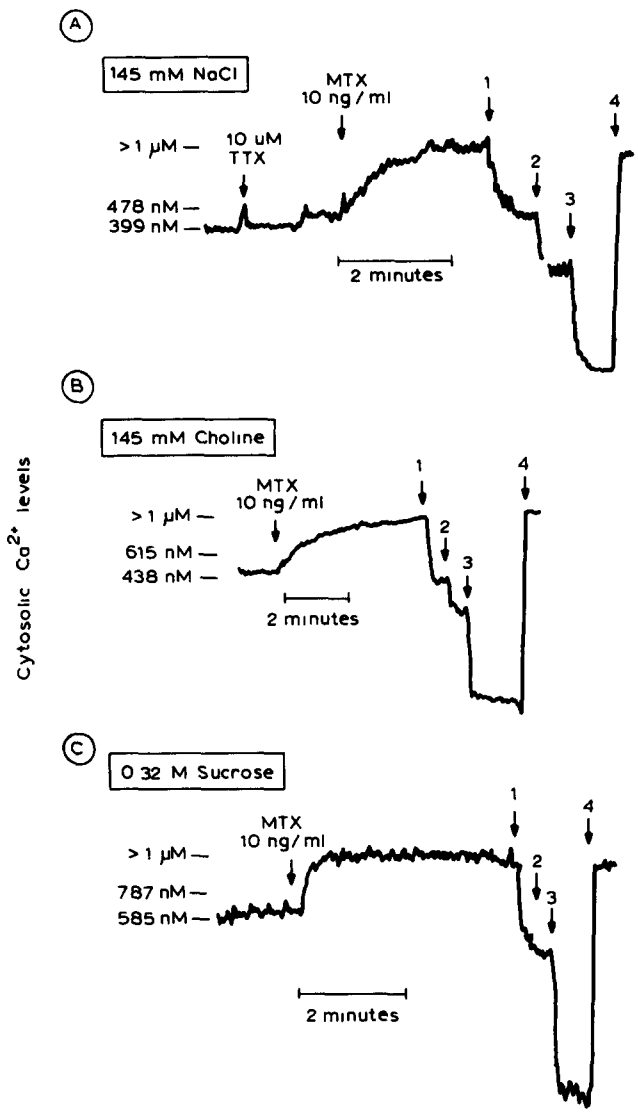

Fig 2 MTX-induced elevation of intrasynaptosomal Ca^{2+} levels is unaffected by TTX, by the substitution of extracellular Na^+ ions with choline and by incubation of synaptosomes in an Na^+-, K^+- and Mg^{2+}-free medium (sucrose substitution) (A) After the equilibration period in presence of 1 mM extracellular Ca^{2+}, synaptosomes were exposed to TTX (10 μM), 2 min before the addition of MTX to the cuvette Calibration of fura-2 fluorescence signal was performed as described in Fig 1A (B) Fura-2-loaded synaptosomes were resuspended, just prior to use, in a standard medium in which Na^+ ions were substituted with an equimolar concentration of choline (145 mM) All the subsequent experimental steps were performed as indicated in the figure Calibration of fura-2 fluorescence signal was performed as described in Fig 1A (C) Fura-2-loaded synaptosomes were resuspended, just prior to use, in a medium in which Na^+, K^+ and Mg^{2+} ions which were present in the normal buffer, were substituted with an isoosmotic concentration of sucrose (0 32 M) All the subsequent experimental steps were performed as indicated in the figure Calibration of fura-2 fluorescence signal was performed as described in Fig 1A

Effect of MTX on intrasynaptosomal Ca^{2+} levels in presence of the Na^+ channel blocker tetrodotoxin or of a Na^+-free choline-substituted medium or of an ion-free sucrose-substituted medium

When the Na^+ channel blocker tetrodotoxin (TTX, 10 μM) was added to the standard Ca^{2+}-containing medium, no modification of either basal or MTX (10 ng/ml)-induced increase of cytosolic Ca^{2+} levels occurred (Fig 2A) Furthermore, since it has been proposed that the stimulation of Ca^{2+} entrance exerted by MTX is dependent on the activation of TTX-insensitive Na^+ channels [19], the effect of MTX (10n g/ml) were studied in a medium in which extracellular Na^+ ions were removed and substituted with an isoosmotic concentration (145 mM) of choline This ionic substitution failed to prevent MTX-induced increase of intrasynaptosomal Ca^{2+} levels (Fig 2B), suggesting that Na^+ entrance through TTX-insensitive Na^+ channels is not a prerequisite for MTX-induced stimulation of Ca^{2+} entrance in brain synaptosomes

In addition, MTX was still able to cause an increase of intrasynaptosomal Ca^{2+} levels when all the extracellular monovalent and divalent cations (except Ca^{2+} ions) were substituted with 0 32 M sucrose, in order to keep constant the medium osmolarity (Fig 2C)

Effect of MTX on bisoxonol-monitored synaptosomal membrane potential

When synaptosomes, incubated in a 1 mM Ca^{2+}-containing standard medium, were exposed to increasing doses (0 1–10 ng/ml) of MTX, a dose-dependent depolarization, monitored by the increase of bisoxonol fluorescence emission, was observed (Fig. 3)

Saturation of the fluorescent signal was observed, at the concentration of 10 ng/ml, within 1 min, and, with a toxin concentration of 3 ng/ml, within 2 min. Lower concentrations of MTX (1 and 0 3 ng/ml), although still able to promote a depolarization of the synaptosomal membrane, did not reach saturability of the fluorescent signal within the time-course followed in the present experiments This was also demonstrated by the ability of a subsequent addition of 50 mM extracellular K^+ ions to further enhance the fluorescence intensity of bisoxonol

Extracellular Ca^{2+}-dependence of MTX-induced synaptosomal membrane depolarization

MTX (10 ng/ml)-induced synaptosomal depolarization was prevented when Ca^{2+} ions were omitted from the external medium, in presence of 1 mM EGTA (Fig. 4A). Furthermore, the reintroduction of an excess of Ca^{2+} ions (5 mM) in the medium, reversed the effect of the Ca^{2+}-chelator and elicited a faster response as compared to that exerted by the toxin in a Ca^{2+}-containing medium (Fig 4B)

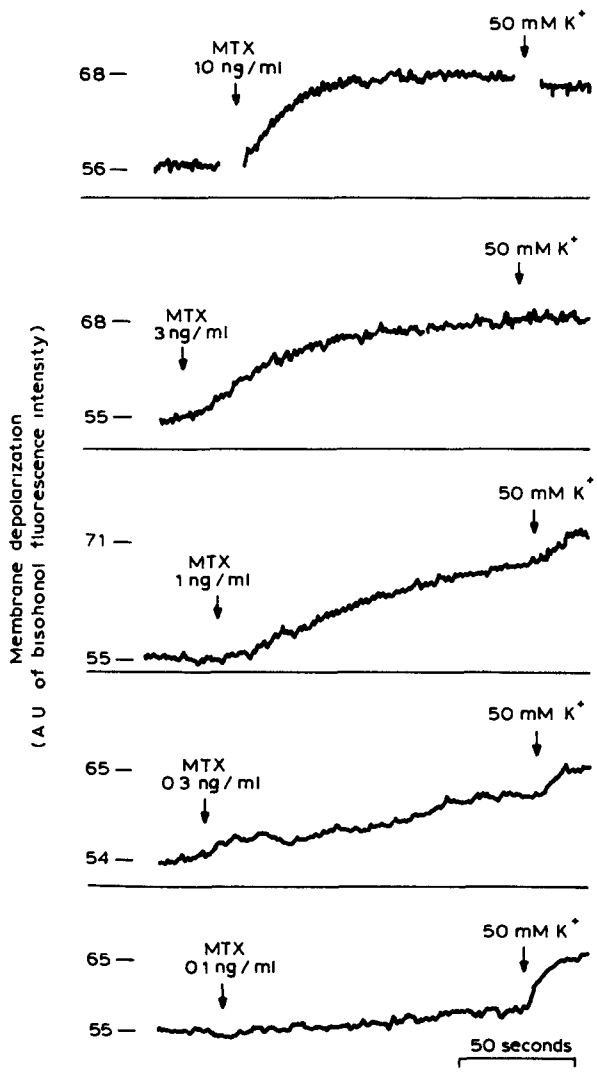

Fig 3 Dose-effect relationship of MTX-induced synaptosomal membrane depolarization After isolation, synaptosomes were resuspended in a standard Ca^{2+}-free medium and subjected to a 30 min incubation at 37°C After this period, they were pelletted, resuspended in the same buffer and kept on ice, until their experimental use Just prior to use, 4–5 μl of synaptosomal resuspension (40–50 μg of synaptosomal protein) was pipetted in the spectrofluorimeter cuvette containing 2 ml of a prewarmed 1 mM Ca^{2+}-containing medium plus 300 nM bisoxonol Fluorescence emission of the dye was recorded for at least 3 min and, in the absence of any other experimental manipulation, it remained stable for the whole duration of the recording. After 3 min, as indicated by the arrow, MTX (0 1–10 ng/ml) was added to the synaptosomes After a further period of 3 min, synaptosomes were exposed to 50 mM extracellular K^+ ions, as indicated by the second arrow The results are expressed as arbitrary units of bisoxonol fluorescence intensity, due to the absence of a reproducible calibration procedure for this fluorescent dye, as described in Materials and Methods

Effect of MTX on synaptosomal membrane potential in presence of the Na^+ channel blocker tetrodotoxin, of a Na^+-free choline-substituted medium or of an ion-free sucrose-substituted medium

TTX (10 μM) was unable to prevent the MTX (10 ng/ml)-induced depolarization of the synaptosomal

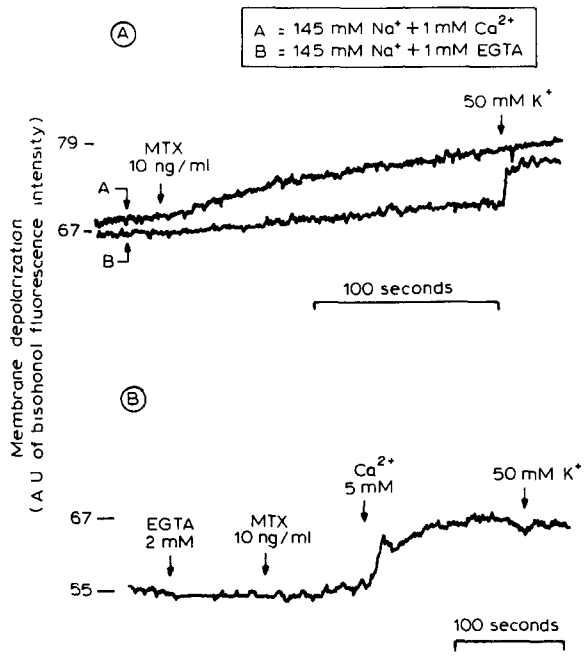

Fig 4 Effect of the removal of extracellular Ca^{2+} ions plus EGTA on MTX-induced depolarization of the synaptosomal membrane The experiment was performed as described in the legend for Fig 3 In Fig 4A, synaptosomes were added in the spectrofluorimeter cuvette containing 2 ml of a prewarmed standard medium in presence of either 1 mM Ca^{2+} ions (Fig 4A, trace A) or 1 mM of the Ca^{2+}-chelator EGTA (Fig 4A, trace B) After 3 min, as indicated by the arrow, MTX (10 ng/ml) was added to the synaptosomes After a further period of 3 min, synaptosomes were exposed to 50 mM extracellular K^+ ions, as indicated by the second arrow In Fig 4B, synaptosomes, after resuspension in a Ca^{2+}-free and EGTA-free medium, were first exposed to EGTA (2 mM) After 2 min, MTX (10 ng/ml) was added to the synaptosomes and, after a further period of 2 min, and Ca^{2+} ions (5 mM) were reintroduced into the medium 3 minutes later, synaptosomes were exposed to 50 mM KCl The results are expressed as arbitrary units of bisoxonol fluorescence intensity, as previously described

membrane (Fig 5A) Furthermore, also when external Na^+ ions were removed and substituted with equiosmolar concentrations of choline, the depolarizing effect exerted by MTX (10 ng/ml) was unaffected (Fig 5B, trace A), whereas, also in the choline-containing medium, the absence of Ca^{2+} ions and the addition of 1 mM of the Ca^{2+}-chelator EGTA prevented MTX-induced depolarization (Fig 5B, trace B) Interestingly, the removal of all extracellular cations except Ca^{2+} ions and their substitution with an isoosmotic concentration of sucrose (0 32 M), did not modify MTX-induced synaptosomal membrane depolarization (Fig 5C, trace A), by contrast, the removal of Ca^{2+} ions and the addition of 1 mM EGTA, completely blocked this effect (Fig 5C, trace B)

Effect of MTX on synaptosomal membrane potential in presence of 1 mM Ba^{2+} or Sr^{2+} ions substituting extracellular Ca^{2+} ions

The substitution of extracellular Ca^{2+} ions with an equivalent concentration (1 mM) of Ba^{2+} or of Sr^{2+}

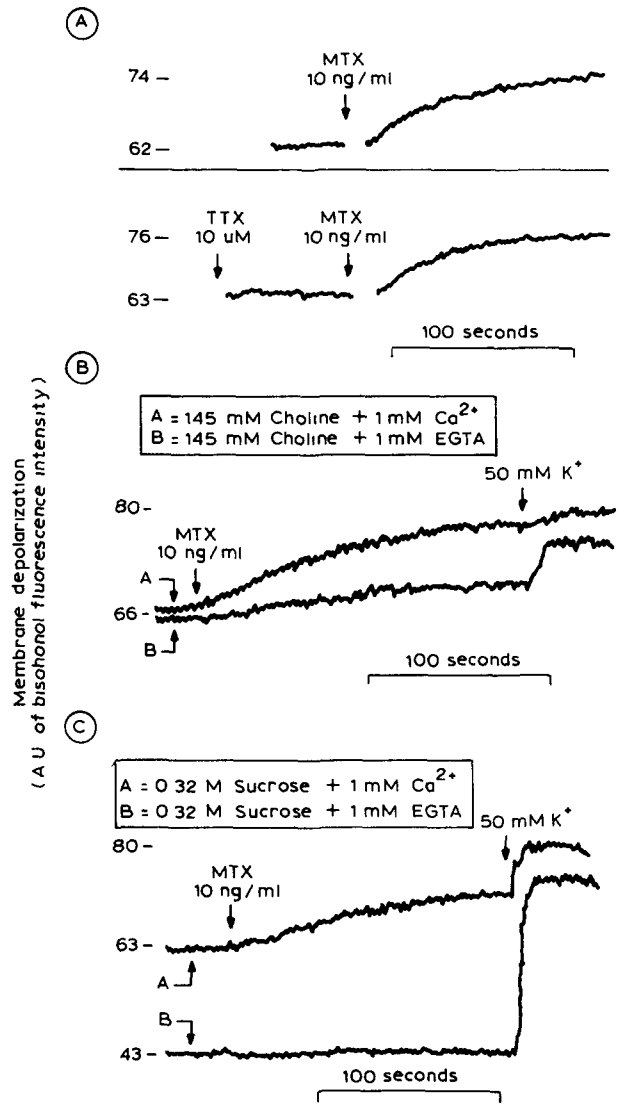

Fig 5 MTX-induced depolarization of the synaptosomal membrane is unaffected by TTX, by the substitution of extracellular Na^+ ions with choline and by incubation of synaptosomes in an Na^+-, K^+- and Mg^{2+}-free medium (sucrose substitution) The experiment was performed as described in the legend for Fig 3 The composition of the extracellular media of three different experiments (A, B and C) was the following (A) standard Ca^{2+}-containing medium, (B) a standard medium in which 145 mM Na^+ was substituted with 145 mM choline and in presence or in absence of Ca^{2+} ions, (C) all the ions were substituted with 0 32 M sucrose, in presence or in absence of Ca^{2+} ions Results are expressed as arbitrary units of bisoxonol fluorescence intensity, due to the absence of a reproducible calibration procedure for this fluorescent dye, as described in Materials and Methods

ions did not modify the depolarizing effect exerted by MTX (10 ng/ml) on synaptosomal membrane potential (Fig 6)

Discussion

The results of the present study suggest that MTX is able to cause a remarkable increase of intrasynapto-

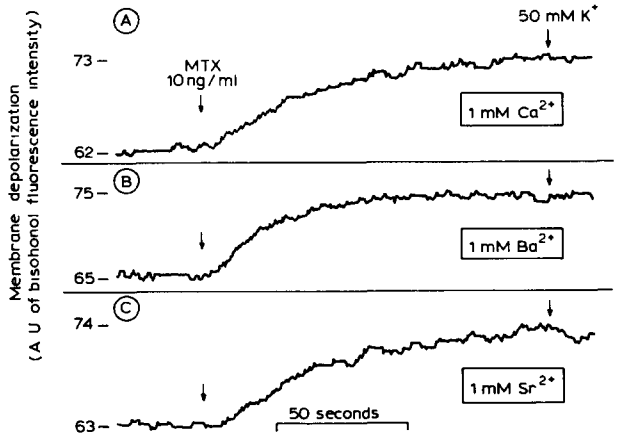

Fig 6 The depolarizing effect of MTX is unaffected by the substitution of extracellular Ca^{2+} ions with an equimolar concentration of Ba^{2+} or of Sr^{2+} ions Synaptosomes were added in the spectrofluorimeter cuvette containing 2 ml of a prewarmed medium containing either 1 mM Ca^{2+} ions (Fig 6A), or 1 mM Ba^{2+} ions (Fig 6B) or 1 mM Sr^{2+} ions (Fig 6C) After 3 min, as indicated by the arrows, MTX (10 ng/ml) was pipetted into the cuvette After a further period of 3 min, synaptosomes were exposed to 50 mM extracellular K^+ ions, as indicated by the second arrow The results are expressed as arbitrary units of bisoxonol fluorescence intensity, as previously described

somal Ca^{2+} levels and to depolarize synaptosomal membrane when added in a standard medium containing Ca^{2+} ions Both these effects of MTX were prevented by the removal of extracellular Ca^{2+} ions, suggesting that the influx of the bivalent cation into the synaptosomes is the event responsible either for the elevation of intrasynaptosomal Ca^{2+} levels and for the depolarizing effect of the toxin Furthermore, the blockade of the MTX-induced depolarization and of the toxin-elicited enhancement of intrasynaptosomal Ca^{2+} levels observed in a Ca^{2+}-free and EGTA-containing medium were both reversed by an excess of extracellular Ca^{2+} ions. Interestingly, the reintroduction of Ca^{2+} ions after the exposure to MTX in a Ca^{2+}-free medium elicited a faster synaptosomal depolarization and increase in intrasynaptosomal Ca^{2+} levels as compared to those occurring when the toxin was added in a medium which already contained Ca^{2+} ions This suggests that toxin binding is the rate-limiting step of both phenomena On the other hand, Na^+ ion entrance does not appear to be implicated in the effects of MTX on synaptosomal Ca^{2+} levels and membrane potential, since both the blockade of TTX-sensitive voltage-operated Na^+ channels and the removal of Na^+ ions, in presence of 1 mM extracellular Ca^{2+}, failed to affect MTX-elicited increase of cytosolic Ca^{2+} levels and MTX-induced depolarization of the synaptosomal membrane These results are in line with those obtained by other authors [4,5], who showed that $^{45}Ca^{2+}$ influx elicited by MTX is unaffected by TTX and that MTX is still effective in evoking neurotransmitter release from NG 108-15 cells under conditions of external Na^+ substitution with choline [9] Furthermore, the fact that MTX depolarizes the synaptosomal plasmamembrane also in the absence of extracellular Na^+ ions is in contrast with the hypothesis of Pin et al [19], who suggested that the depolarizing effects of MTX are dependent on the activation of TTX-insensitive Na^+ channels and that MTX-induced Ca^{2+} entrance in cultured striatal neurons is consequent to the depolarizing effect due to the activation of these Na^+ channels

The prevalent role played by Ca^{2+} entrance in the depolarizating effect exerted by MTX, emerged from the experiments in which the complete removal of external cations, including Ca^{2+}, and their substitution with 0 32 M sucrose completely blocked MTX-induced depolarization Under both the mentioned experimental conditions, the reintroduction of only Ca^{2+} ions in the extracellular medium restored the depolarizing effect of MTX These results strongly supported the idea that MTX caused an increase of intrasynaptosomal Ca^{2+} levels through an enhancement of extracellular Ca^{2+} influx, and that this influx induces membrane depolarization

This view seems to be supported by the patch-clamp experiments recently performed by Kobayashi et al [20] in guinea-pig cardiac cells showing that MTX activates a sustained inward current, with Ca^{2+} being the dominant charge carrier of this current It should be underlined that, also in neuroblastoma cells [21], using a conventional intracellular recording technique, it has been demonstrated that MTX produced a depolarization of the membrane which was prevented by the removal of Ca^{2+} ions from the external medium

Furthermore, the possibility that the synaptosomal depolarization induced by MTX may derive from a nonspecific membrane damage subsequent to the activation of Ca^{2+}-dependent lipases or proteases seems unlikely, since the substitution of Ca^{2+} ions with other bivalent cations like Ba^{2+} or Sr^{2+} ions did not modify the depolarizing effect exerted by MTX on synaptosomal membrane potential

In conclusion, the results of the present study strenghtens the view that MTX is able to increase cytosolic Ca^{2+} levels in brain synaptosomes through an activation of a Ca^{2+} conductance at the synaptosomal membrane level which is independent on the presence of Na^+ ions in the extracellular medium This massive entrance of Ca^{2+} ions into the synaptosomes causes a depolarization of the synaptosomal membrane

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