0026-895X/94/020300-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:300-305

Maitotoxin Activates a Nonselective Cation Channel and Stimulates Ca^{2+} Entry in MDCK Renal Epithelial Cells

PAUL DIETL and HARALD VÖLKL

Department of Physiology, University of Innsbruck, A-6020 Innsbruck, Austria

Received July 23, 1993; Accepted November 16, 1993

SUMMARY

We examined the mechanisms of maitotoxin (MTX), a water-soluble polyether from the marine dinoflagellate *Gambierdiscus toxicus*, in stimulation of Ca^{2+} entry into Mardin-Darby canine kidney cells. In the presence of bath Ca^{2+}, MTX (3 nM) caused an elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$), which was partially inhibited by SK&F 96365 (25 μM) or La^{3+} (100 μM). A stimulation of Ca^{2+}-dependent K^+ channels in cell-attached membrane patches coincided with this rise in $[Ca^{2+}]_i$ and was also partially inhibited by SK&F 96365. Before the rise in $[Ca^{2+}]_i$, a nonselective cation current (I_{ns}), studied by the whole-cell patch-clamp technique, was irreversibly activated. I_{ns} poorly discriminated between Na^+, K^+, and Cs^+, was unaffected by replacement of Cl^- with gluconate$^-$, and was not voltage gated. MTX-induced I_{ns} was partially blocked by La^{3+} ions (100 μM) but not by SK&F 96365 (25 μM) or nifedipine (10 μM). SK&F 96365 by itself induced a small but significant stimulation of I_{ns} and a rise in $[Ca^{2+}]_i$. The activation of I_{ns} by MTX was instantaneous and depended on the presence of extracellular Ca^{2+} ions. In the absence of other cations, the inward current of I_{ns} was dependent on the bath Ca^{2+} concentration. Cell-attached and excised single-channel measurements revealed that MTX activated a SK&F 96365-insensitive, ≈40-pS, nonselective cation channel from the outside. We conclude that the initial action of MTX is the stimulation of a nonselective cation channel, which requires the presence of extracellular Ca^{2+} ions. The subsequent rise in $[Ca^{2+}]_i$ is at least in part caused by another, SK&F 96365-sensitive, Ca^{2+} entry pathway, which may be activated as a result of or independently of I_{ns}.

In the search for chemical tools that modulate the function of ion channels, MTX has attracted interest because of its ability to stimulate Ca^{2+} entry into various excitable and nonexcitable cell types (1). The mechanism involved therein, however, is still a matter of debate. In several electrophysiological studies, cell membrane depolarization or an inward current in response to MTX was observed (2–5). In neuroblastoma cells, MTX induced a Ca^{2+}-dependent cell membrane depolarization, whereas Ba^{2+} currents through VDCC gradually declined (3). A critical dependence of the membrane depolarization on Ca^{2+} was also reported in purified rat brain synaptosomes, whereas substitution of Na^+ with choline$^+$ did not prevent the depolarization (5). In cardiac cells, the MTX-induced inward current exhibited an almost linear current-voltage relationship, indicating that it was voltage independent, and was enhanced by adrenaline (4). Depolarization or inward currents may result from rheogenic Ca^{2+} entry itself or from activation of Ca^{2+}-dependent processes that depolarize the cell membrane. Ca^{2+} entry may further involve a VDCC, NSCIC, receptor-operated channel, second messenger-gated channel, or calcium release-activated channel (6, 7). Relevant to this question, one theory claims that the initial step of Ca^{2+} entry stimulated by MTX occurs through L-VDCC and then triggers a variety of secondary events, sustaining the elevation of $[Ca^{2+}]_i$ (2). Direct electrophysiological evidence for the activation of L-VDCC by MTX, however, could not be obtained (3). In other studies, MTX appeared to be associated with receptor-mediated Ca^{2+} entry (8) or Ca^{2+} entry through another class of Ca^{2+} channels (4). Importantly, MTX was shown to lack ionophoretic activity (9, 14). In addition, MTX causes phosphoinositide breakdown (8) and release of neurotransmitters (2, 9).

In view of the different cellular events that are activated by MTX, the question remains which event comes first, i.e., what is the molecular target site of MTX? We combined measurements of free $[Ca^{2+}]_i$ using the Ca^{2+} indicator fluo-3 with whole-cell and single-channel current measurements and compared the time course of the Ca^{2+} response with that of current responses observed upon activation with MTX. Aiming at a least complex model, the role of L-VDCC in the action of MTX was excluded by using epithelial cells, which do not express L-VDCC (10). Our data indicate that MTX initially activates a

This study was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung (Grant P9101-MED) and the Legerlotz-Stiftung.

ABBREVIATIONS: MTX, maitotoxin; I_{ns}, nonselective current; NSCIC, nonselective cation channel; VDCC, voltage-dependent calcium channel(s); L-VDCC, L-type voltage-dependent calcium channel(s); V_{rev}, reversal potential; $[Ca^{2+}]_i$, intracellular calcium concentration; SK&F 96365, 1-[3-(4-methoxyphenyl)propoxyl]-1-(4-methoxyphenyl)ethyl-1*H*-imidazole HCl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; MDCK, Mardin-Darby canine kidney.

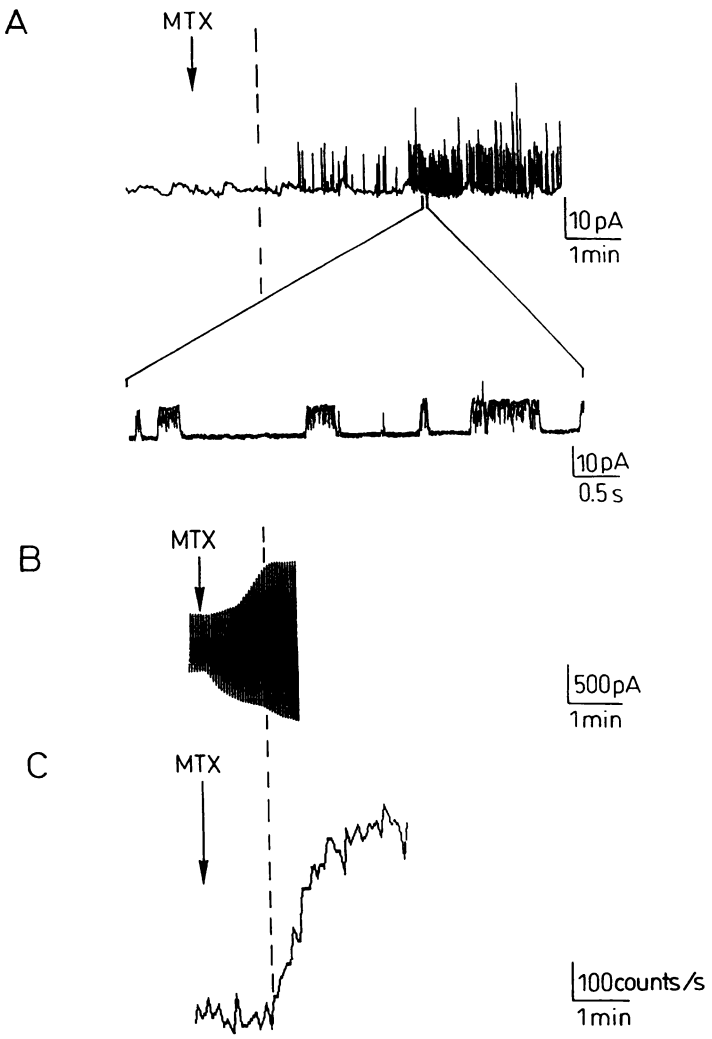


Fig. 1. Temporal comparison of MTX (3 nM) effects on the Ca^{2+}-activated K^+ channel (cell-attached patch) (A), on the whole-cell current (repetitive voltage ramps from −60 mV to 60 mV) (B), and on the fluo-3 signal (C). Original tracings from three representative experiments are shown. The time scales in A, B, and C are identical; MTX addition is synchronous in A, B, and C. *Dashed line*, onset of the $[Ca^{2+}]_i$ increase in all traces, for easier comparison. In A, upward deflections represent brief openings of the Ca^{2+}-activated K^+ channel (see also expanded time scale). In B, note the immediate increase of the whole-cell current upon MTX addition.

Ca^{2+}-conducting, non-voltage-gated NSCIC, which requires Ca^{2+} at the extracellular site. In other cell types, the depolarization induced by I_{Na} could account for the activation of L-VDCC.

Materials and Methods

MDCK cells (passages 70–100) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 26 mM $NaHCO_3$. Cells were grown at 37° in 5% CO_2-containing humidified air. For the experiments, the cells were seeded at low density on a plastic Petri dish and single cells were patched 1–3 days later at a subconfluent state. Perfusion of the cells was performed via a multichannel pipette, allowing rapid exchanges of the bath solution. The control bath solution contained 140 mM NaCl, 10 mM Tris, 10 mM glucose, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4 (with HCl). Ca^{2+}-free bath solutions contained no Ca^{2+} but included 1 mM EGTA. For the whole-cell experiments, the pipette solution contained 140 mM potassium gluconate, 2 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, and 10 mM Tris, pH 7.4 (with HCl).

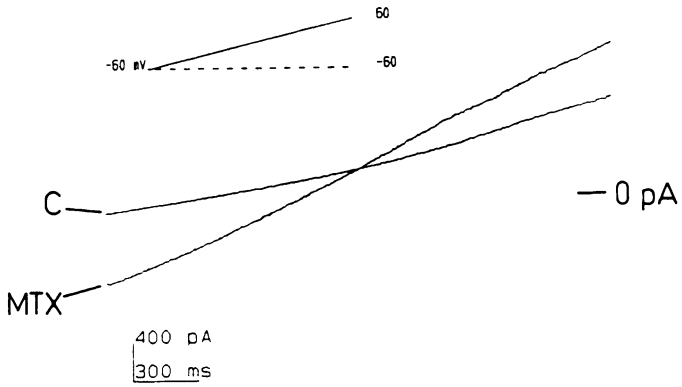


Fig. 2. Expanded superimposed whole-cell current traces of a representative experiment in response to voltage ramps (−60 mV to 60 mV), as in Fig. 1B. *C*, Control bath solution; *MTX*, control bath solution plus 3 nM MTX. Traces were recorded after a steady state had been reached under control and experimental conditions. Activation by MTX did not considerably differ in time from that shown in Fig. 1B.

Whole-cell patch-clamp experiments were performed as described previously (11), with minor modifications. In general, repetitive voltage ramps (20/min) of 2.3-sec duration each were performed from a holding potential of −60 mV to 60 mV, using a pCLAMP 5.5 routine. The currents were measured with an EPC-7 amplifier and stored at a sampling rate of 220 Hz on the hard disk of a 386SX computer. Single-channel currents were stored on a VHS tape after conversion by a pulse code modulation recorder (Sony 501ES). For analysis, a representative record of >1 min for each experimental protocol was filtered at 1 kHz using a four-pole Bessel filter and was sampled at a rate of 5000 Hz on the hard disk of a 386SX computer, using a pCLAMP routine. Channels were considered open when the current was larger than $i/2$. The probability of a channel being open (P_o) was defined as the total time the channel was in the open state divided by the total time of data collection.

Fluo-3 fluorescence has been used for determination of $[Ca^{2+}]_i$. Glass coverslips with cells were incubated for 1 hr in Dulbecco's modified Eagle's medium containing fluo-3/AM (3 μM) and pluronic F-127 (20% in dimethylsulfoxide) (3 μM fluo-3/AM/pluronic F-127 stock, 9:1). Pluronic F-127 is a nonionic polyol (molecular weight, approximately 12,500) that facilitates the solubilization of water-insoluble dyes. Coverslips were then placed into a perfusion chamber containing the control bath solution (see above) and measurements were made under a converted phase-contrast microscope (Zeiss IM-35) equipped with a photomultiplier tube (R4829; Hamamatsu, Herrsching, Germany). Fluorescense was monitored at 498 nm (excitation) and 520 nm (emission). $[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz *et al.* (12). Maximal fluorescence (R_{max}) was determined by exposing the cells to either 20 μM A23187 or 20 μM digitonin; minimal fluorescense (R_{min}) was determined by exposure to 2 mM Mn^{2+} and 20 μM A23187. Mean values of R_{max} and R_{min} were used to calculate $[Ca^{2+}]_i$ in a nonpaired fashion.

MTX was purchased from Wako Chemicals (Neuss, Germany). Fluo-3/AM and pluronic F-127 were purchased from Molecular Probes (Eugene OR). SK&F 96365 was kindly provided by SKB Pharmaceuticals (Surrey, UK). All other chemicals were purchased from Sigma (Munich, Germany).

Data are reported as means ± standard errors. Student's t test was used for statistical analysis. Differences between means were considered significant at p values of <0.05.

Results

Two lines of evidence indicate that MTX stimulated Ca^{2+} entry into MDCK cells. 1) $[Ca^{2+}]_i$ was significantly increased from 83 ± 12 nM to 430 ± 95 nM upon stimulation with MTX (3 nM). This rise in $[Ca^{2+}]_i$ was dependent on bath Ca^{2+} and

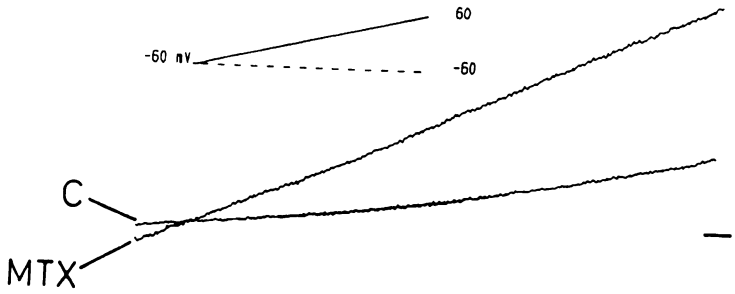


Fig. 3. Current tracings as described in Fig. 2. *C*, NaCl-free bath solution, with 280 mM mannitol replacing 140 mM NaCl; *MTX*, NaCl-free bath solution plus 3 nM MTX.

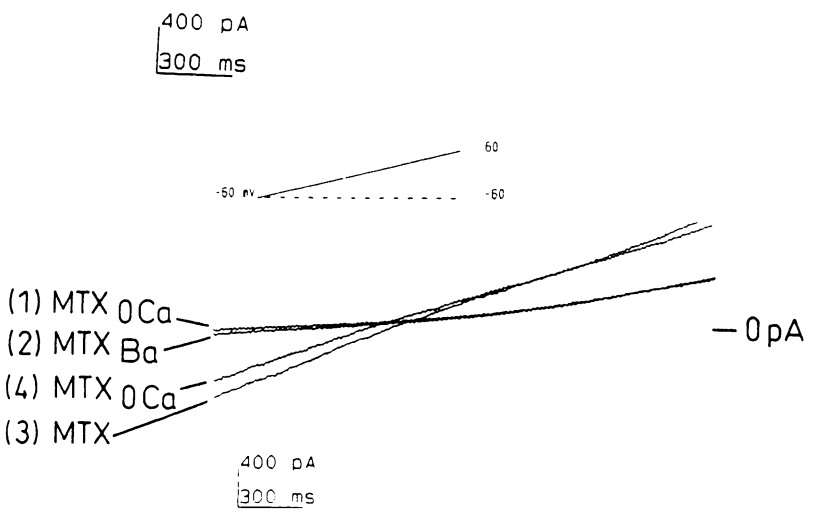


Fig. 4. Current tracings as described in Fig. 2. *1-4*, Sequence of the experimental protocol (*1* first, *4* last). MTX_{0Ca}, control Ca^{2+}-free bath solution (0 mM Ca^{2+}, 1 mM EGTA) plus 3 nM MTX. *1*, Ca^{2+}-free bath solution plus 3 nM MTX was given as the first experimental solution and the ramp current was the same as with a MTX-free, Ca^{2+}-free bath solution. *4*, Ca^{2+}-free bath solution plus 3 nM MTX was given at the end of the experiment, i.e., after I_{ns} activation by MTX plus Ca^{2+}. MTX_{Ba}, control bath solution with 1 mM $BaCl_2$ replacing 1 mM $CaCl_2$, plus 3 nM MTX. *MTX*, control bath solution plus 3 nM MTX.

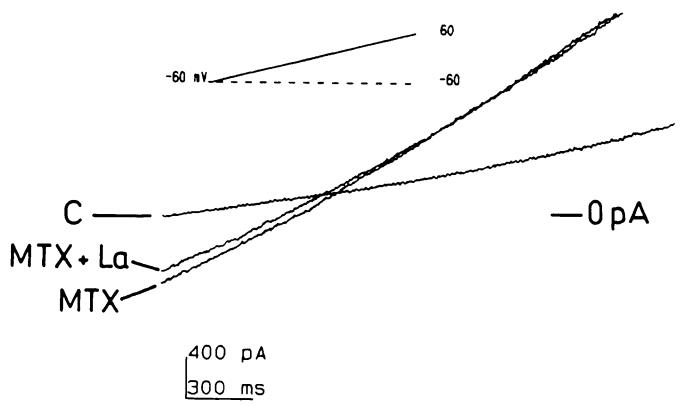


Fig. 5. Current traces as described in Fig. 2. *C*, Control bath solution; *MTX*, control bath solution plus 3 nM MTX; *MTX + La*, control bath solution plus 3 nM MTX plus 0.1 mM La^{3+}.

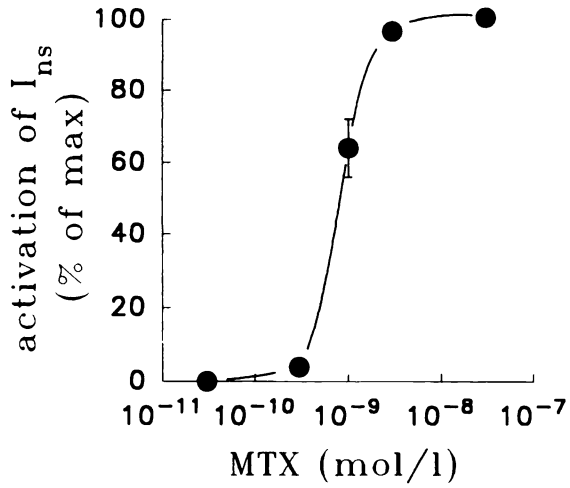


Fig. 6. Dependence of the MTX concentration on the activation of I_{ns}. Maximum activation was arbitrarily defined as activation at a MTX concentration of 30 nM. $I_{MTX} - I_{control}$ at −60 mV was taken as a parameter for I_{ns}. Slope factor = 3.06, EC_{50} = 824 pM.

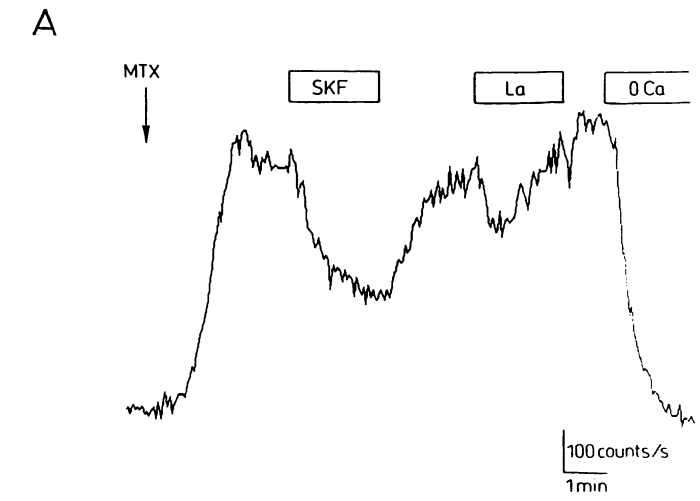
TABLE 1

Dependence of MTX-induced I_{ns} at −60 mV ($I_{MTX} - I_{control}$) on different bath ion concentrations

Bath ions	I_{ns}	*n*
mM	*pA*	
140 Na^+, 1 Ca^{2+}	860 ± 292	32
140 Cs^+, 1 Ca^{2+}	792 ± 263	15
140 K^+, 1 Ca^{2+}	943 ± 303	6
280 Mannitol, 1 Ca^{2+}	52 ± 14	5
253 Mannitol, 10 Ca^{2+}	186 ± 66	7

was delayed by >1 min after MTX application (Fig. 1C). 2) MTX stimulated single Ca^{2+}-dependent K^+ channels in cell-attached patches (Fig. 1A). We made use of outwardly rectifying K^+ channels, which are quiescent in unstimulated MDCK cells and become active upon stimulation with agonists, mediating an elevation of $[Ca^{2+}]_i$ (13). MTX caused a significant increase of the P_o of this channel from 0 to 0.047 ± 0.032 (n = 9; in one of nine experiments no channel activity could be elicited). The time course of K^+ channel activation roughly equaled that of the $[Ca^{2+}]_i$ rise, and activation was also dependent on bath Ca^{2+} (Fig. 1). Removal of bath Ca^{2+} reduced the MTX-induced P_o from 0.052 ± 0.028 to 0.006 ± 0.005 (n = 3). This suggests that MTX activated K^+ channels via Ca^{2+} influx.

To further evaluate the mechanisms of MTX-induced Ca^{2+} entry, whole-cell patch-clamp experiments were performed.



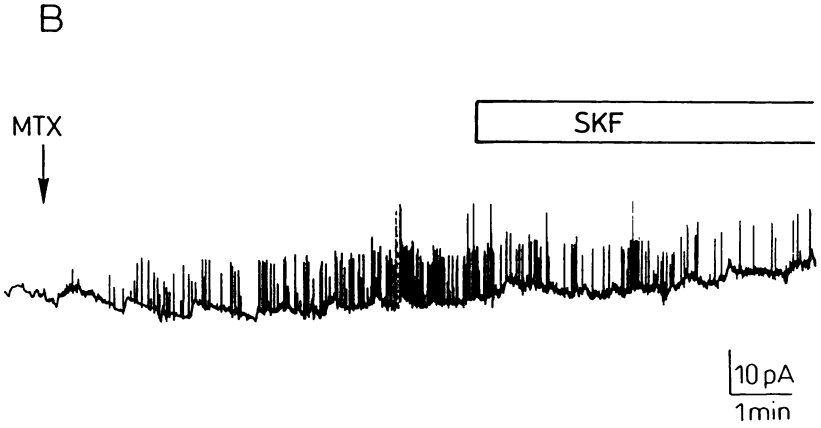


Fig. 7. A, Fluo-3 signal of a representative experiment in response to 3 nM MTX. Addition of 25 μM SK&F 96365 or 0.1 mM La^{3+} to the MTX-containing solution is indicated *above the tracing*. *0 Ca*, removal of bath Ca^{2+} (1 mM EGTA). B, Original cell-attached patch-clamp recording of a Ca^{2+}-activated K^+ channel as described in Fig. 1A. MTX concentration, 3 nM; SK&F 96365 concentration, 25 μM.

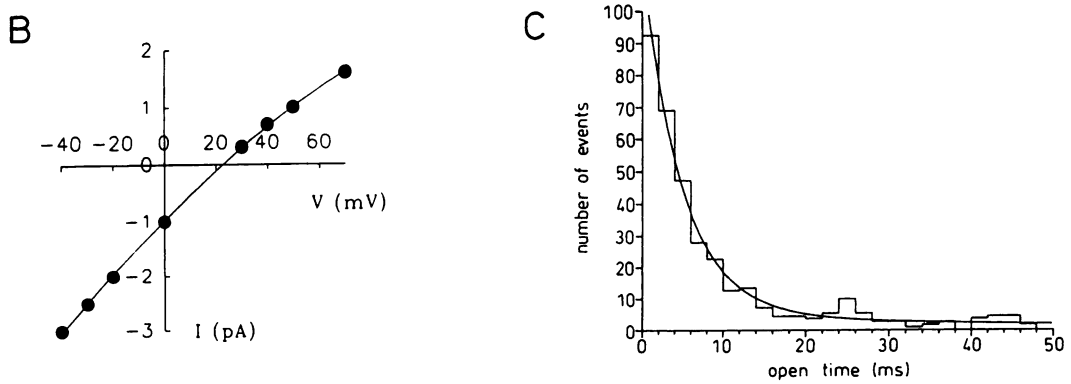


Fig. 8. A, Cell-attached single-channel recording of three NSCICs/patch. Three channels instead of three substates of one channel were assumed because levels 2 and 3 are multiples of level 1 and transitions from the closed state to level 3 were never observed. The pipette was filled with control bath solution containing 30 nM MTX. B and C, Current-voltage relationship (B) and open time histogramm (C) of a single channel.

With nearly physiological bath and pipette solutions, MTX consistently increased the cell conductance, with the onset of the increase being only a few seconds after the addition of MTX (Fig. 1B). Voltage ramps from −60 mV to 60 mV were performed to gain insight into the V_{rev} values of the MTX-induced currents. In Figs. 2-5, expanded current traces in response to voltage ramps are shown. All of these traces were recorded at a time when a steady state had been reached under control and experimental conditions. MTX shifted the V_{rev} from −56 ± 6 mV to −9.3 ± 12 mV (n = 16). Because the theoretical V_{rev} of each ion differs considerably from this value (K^+, −∞; Na^+, ∞; Ca^{2+}, ∞; Cl^-, −65 mV), a relatively nonselective current (I_{ns}) was activated by MTX. For further analysis, $I_{MTX} - I_{control}$ at −60 mV was taken as a parameter for the MTX-induced I_{ns}, because at this voltage errors arising from an eventual change of the Cl^- conductance were kept to a minimum. Values with various bath ions substituting for Na^+ are listed in Table 1. The results show that I_{ns} discriminated poorly between Na^+, K^+, and even Cs^+. A concentration-response curve for MTX activating I_{ns} is shown in Fig. 6. To exclude the possibility that I_{ns} was voltage gated, i.e., that it was induced by our voltage ramp protocol, the potential was continuously held at −80 mV and MTX was applied. I_{ns} was as well activated and did not change in magnitude when voltage ramps were subsequently performed (n = 4).

Does MTX-induced I_{ns} also carry Ca^{2+}? This question was addressed by replacing bath Na^+ and Mg^{2+} with mannitol, leaving Ca^{2+} as the only cation in the bath (except for protonated Tris). Fig. 3 shows that even under these conditions MTX caused a small inward current at −60 mV, which reversed at −54.2 ± 5.7 mV (n = 5), and a large outward current at more positive potentials, representing K^+ efflux. Moreover, the inward current at −60 mV was dependent on the bath Ca^{2+} concentration (Table 1). These data indicate that Ca^{2+} contributed to I_{ns}.

Is I_{ns} a Ca^{2+}-activated current? This question was addressed by applying MTX to a Na^+-containing bath solution before addition of Ca^{2+}. Fig. 4 shows that in the absence of Ca^{2+} MTX was almost entirely ineffective; I_{ns} averaged only 2.1 ± 2.4% of the response seen after Ca^{2+} addition (n = 12). Conversely, when I_{ns} was fully activated by Ca^{2+} and MTX, Ca^{2+} removal at a later time only partially reversed this activation, leading to only a small and insignificant reduction of I_{ns} within 15 min (Fig. 4). These data indicate that the activation of I_{ns} by MTX is a Ca^{2+}-dependent process. Once initiated, however, this effect is irreversible. Other divalent cations (Ba^{2+}, n = 6, or Mn^{2+}, n = 2) were not able to induce I_{ns} (Fig. 4).

Are there specific inhibitors of MTX-induced I_{ns}? The actions of MTX have been associated with L-VDCC- (2) or receptor-mediated (8) Ca^{2+} entry. We tested nifedipine (10 μM), La^{3+} (100 μM), and the imidazole derivative SK&F 96365 (25 μM). Among these compounds, only La^{3+} exerted a small but significant inhibition of the MTX-stimulated I_{ns}, of 5.4 ± 2.8% (n = 7) (Fig. 5). Nifedipine and SK&F 96365 did not inhibit I_{ns} (n = 4 and 8, respectively). All of these drugs were given about 10 sec after MTX reached its maximum stimulation of I_{ns} (after about 1.5–2 min). The effects of La^{3+} and SK&F 96365 on $[Ca^{2+}]_i$ were also examined (Fig. 7A). Both compounds partially reversed MTX-elevated $[Ca^{2+}]_i$, La^{3+} transiently by 17 ± 6% and SK&F 96365 by 61 ± 15%. Hence, the reduction of $[Ca^{2+}]_i$ by La^{3+} well matched its inhibition of I_{ns}, whereas the effect of SK&F 96365 on $[Ca^{2+}]_i$ was apparently unrelated to I_{ns}. La^{3+} had no significant effect on $[Ca^{2+}]_i$ in the absence of MTX (n = 4); however, SK&F 96365 significantly increased the resting $[Ca^{2+}]_i$ from 108 ± 14 nM to 136 ± 22 nM (n = 5). Addition of MTX after La^{3+} dramatically increased $[Ca^{2+}]_i$ to 430 ± 126 nM (n = 3). After SK&F 96365, the rise in $[Ca^{2+}]_i$ was less pronounced (to 253 ± 77 nM, n = 3) but still significant. Thus, pretreatment with these drugs did not prevent the action of MTX. The reduction of MTX-elevated $[Ca^{2+}]_i$ by SK&F 96365 is in agreement with its inhibition of the MTX-activated Ca^{2+}-dependent K^+ channel (Fig. 7B). In four experiments, SK&F 96365 significantly reduced the P_o from 0.068 ± 0.027 (MTX) to 0.039 ± 0.024 (MTX plus SK&F 96365).

SK&F 96365 exerted a small stimulatory effect on I_{ns} by itself (i.e., in the absence of MTX). The current stimulated by 25 μM SK&F 96365 exhibited a V_{rev} of −4.8 ± 4.6 mV (n = 5) and averaged 185 ± 66 pA (−60 mV).

To characterize the target of MTX on the single-channel level, MTX (30 nM) was added to the patch pipette. To ensure that the observed channels were not spontaneously active, the tip of the pipette was filled with MTX-free control bath solution and the pipette was back-filled with MTX-containing solution. Thus, MTX had to diffuse to the pipette tip before it could activate a channel. In seven of 22 cell-attached patches, two or more NSCICs/patch, which were never spontaneously active upon gigaseal formation, were activated after a delay of 0–5 min. In the remaining 15 patches, no activity was observed for 15 min. This suggests that MTX activated clusters of channels within the membrane. The channel properties are shown in Fig. 8, with the single-channel conductance being about 40 pS. The nonselectivity of the channel was verified by excising the patch and replacing bath Na^+ by K^+ and Cl^- by gluconate$^-$. Neither procedure affected the V_{rev} around 0 mV.

To gain more insight into the mechanism of the NSCIC activation and to confirm the sidedness of the MTX effect, excised membrane patches were studied. With exchanged bath and pipette solutions (i.e., the "intracellular" solution was bath solution), addition of MTX (30 nM) to the bath did not induce channel activity in 13 "silent" inside-out patches. On the other hand, when excised silent outside-out patches were studied, channel activity was induced in four of 14 patches with a delay of a few seconds upon addition of MTX. As in the cell-attached patches, more than one channel per patch was present if active. Addition of 25 μM SK&F 96365 did not reduce the number of active channels in the patch (n = 4). These data confirm that the target of MTX is facing the extracellular side of the membrane. They suggest a direct activation of the NSCIC by MTX and argue against a soluble factor or the cell volume mediating the activation of the NSCIC.

Discussion

The following novel findings are presented in this study. 1) The activation of a NSCIC by MTX is not a result of Ca^{2+} entry but, instead, Ca^{2+} entry lies downstream in the cascade of MTX-induced events. The activation of NSCIC precedes the appearance of all other described actions of MTX. 2) MTX requires extracellular Ca^{2+} ions to activate the NSCIC. This process is irreversible.

The present study confirms earlier observations that MTX stimulates Ca^{2+} entry and causes a sustained cell membrane depolarization by activation of I_{ns} (2–5). It is also consistent with the finding that MTX enhances the permeability of various mono- and divalent cations (14). More ambiguous is the

question of the Ca^{2+} dependence of the MTX effect. MTX could require Ca^{2+} at an intracellular or extracellular site to stimulate I_{ns}. The following arguments strongly suggest that it is indeed the extracellular site at which Ca^{2+} is required. 1) The activation of I_{ns} preceded the increase of $[Ca^{2+}]_i$ (Fig. 1). This alone excludes the possibility that Ca^{2+} was a "second messenger" for MTX, although it does not exclude the possibility that MTX required some intracellular "threshhold Ca^{2+}" to exert its effect. 2) When the bath solution was exchanged from 1 mM Ca^{2+} to 0 mM Ca^{2+} plus MTX, no stimulation of I_{ns} could be seen. Thus, in the absence of bath Ca^{2+} MTX was ineffective, even though $[Ca^{2+}]_i$ was still at base-line levels. The same was true for the reverse procedure; when Ca^{2+} was added to a Ca^{2+}-free, MTX-containing bath, I_{ns} appeared instantaneously (within a few seconds), much too fast for $[Ca^{2+}]_i$ to reach its new level. 3) A high pipette EGTA concentration (10 mM) did not prevent the stimulatory effect of MTX on I_{ns} ($n = 3$). We therefore hypothesize that Ca^{2+} and MTX both have an extracellular binding site at the NSCIC and that both must be occupied for activation by MTX. Because MTX did not alter the seal resistance of single-channel patches and did not increase the whole-cell conductance when applied intracellularly ($n = 2$), our study confirms earlier findings that MTX lacks ionophoretic activity (9, 14).

A puzzling finding is the lack of effect of SK&F 96365 on the MTX-induced I_{ns} but its partially inhibitory action on Ca^{2+} entry. As a clear consequence thereof, I_{ns} cannot be the sole source for the MTX-induced Ca^{2+} entry and it seems that another Ca^{2+} entry pathway (or even more than one) is further activated by MTX. The other pathway may be activated as a result of or independently of, however subsequent to, the activation of I_{ns}. SK&F 96365 partially inhibited the MTX-induced elevation of $[Ca^{2+}]_i$ at a concentration known to entirely block "receptor-mediated" Ca^{2+} entry (15); this suggests that I_{ns} may have contributed to the remaining portion of Ca^{2+} entry. A recent study demonstrated an inhibitory effect of SK&F 96365 on ATP- and *N*-formyl-L-methionyl-L-lencyl-L-phenylalanine-stimulated cation currents (16). This is different from the apparently electroneutral action of SK&F 96365 on MTX-mediated Ca^{2+} entry in our study. Because in addition the SK&F 96365-sensitive NSCIC exhibits a far smaller conductance (4–5 pS) than does the MTX-activated NSCIC, they seem to be entirely different channels. Paradoxically, in our study SK&F 96365 activated I_{ns} and Ca^{2+} entry in the absence of MTX at the same concentration used to inhibit Ca^{2+} entry in the presence of MTX. The same observation (i.e., activation of I_{ns} and Ca^{2+} entry by SK&F 96365) was made by Schwarz *et al.* (17) in endothelial cells, although higher concentrations of SK&F 96365 were needed. At present we do not know which electroneutral Ca^{2+} entry pathway may be inhibited by SK&F 96365, nor do we know the mechanism by which SK&F 96365 activates the NSCIC. Recent evidence suggests that the SK&F 96365-sensitive Ca^{2+} entry pathway may be in some way linked to the activation of phospholipase C by MTX (8).

Although evidence suggests an involvement of L-VDCC in the action of MTX (2), our data exclude the possibility that the presence of L-VDCC is a *conditio sine qua non* for the action of MTX. We also can rule out the possibility that MTX may have activated "quiescent" L-VDCC; the stimulation of I_{ns} at hyperpolarized membrane potentials and the inability of nifedipine to prevent this stimulation clearly argue against such a possibility. It is possible that in electrically excitable tissues the activation of L-VDCC by MTX occurs indirectly, i.e., via depolarization by I_{ns}.

In conclusion, our study suggests a direct activation of the NSCIC by MTX. MTX may thus prove to be a valuable tool in the further characterization of this class of channels.

Acknowledgments

The authors thank Drs. J. Strießnig and E. Wöll for helpful comments and suggestions throughout this study and Dr. J. W. Daly for providing information about MTX. The technical assistance of G. Siber, H. Heitzenberger, and S. David is gratefully acknowledged.

References

1. Gusovsky, F., and J. W. Daly. Maitotoxin: a unique pharmacological tool for research on calcium-dependent mechanisms. *Biochem. Pharmacol.* **39**:1633–1639 (1990).
2. Xi, D., F. M. Van Dolah, and J. S. Ramsdell. Maitotoxin induces a calcium-dependent membrane depolarization in GH_4C_1 pituitary cells via activation of type L voltage-dependent calcium channels. *J. Biol. Chem.* **267**:25025–25031 (1992).
3. Yoshii, M., A. Tsunoo, Y. Kuroda, C. H. Wu, and T. Narahashi. Maitotoxin-induced membrane current in neuroblastoma cells. *Brain Res.* **424**:119–125 (1987).
4. Kobayashi, M., R. Ochi, and Y. Ohizumi. Maitotoxin-activated single calcium channels in guinea-pig cardiac cells. *Br. J. Pharmacol.* **92**:665–671 (1987).
5. Taglialatela, M., L. M. T. Canzoniero, A. Fatatis, G. Di Renzo, T. Yasomoto, and L. Annunziato. Effect of maitotoxin on cytosolic Ca^{2+} levels and membrane potential in purified rat brain synaptosomes. *Biochim. Biophys. Acta* **1026**:126–132 (1990).
6. Putney, J. W. Capacitative calcium entry revisited. *Cell Calcium* **11**:611–624 (1990).
7. Irvine, R. F. Inositol phosphates and Ca^{2+} entry: toward a proliferation or a simplification. *FASEB J.* **6**:3085–3091 (1992).
8. Soergel, D. G., T. Yasumoto, J. W. Daly, and F. Gusovsky. Maitotoxin effects are blocked by SK&F 96365, an inhibitor of receptor-mediated calcium entry. *Mol. Pharmacol.* **41**:487–493 (1991).
9. Takahashi, M., M. Tatsumi, Y. Ohizumi, and T. Yasumoto. Ca^{2+} channel activating function of maitotoxin, the most potent marine toxin known, in clonal rat pheochromocytoma cells. *J. Biol. Chem.* **258**:10944–10949 (1983).
10. Lang, F., F. Friedrich, M. Paulmichl, W. Schobersberger, A. Jungwirth, M. Ritter, M. Steidl, H. Weiss, E. Wöll, E. Tschernko, R. Paulmichl, and C. Hallbrucker. Ion channels in Mardin-Darby canine kidney cells. *Renal Physiol. Biochem.* **13**:82–93 (1990).
11. Dietl, P., N. Kizer, and B. A. Stanton. Conductive properties of a rabbit cortical collecting duct cell line: regulation by isoproterenol. *Am. J. Physiol.* **262**:F578–F582 (1992).
12. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescense properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
13. Kolb, H.-A., M. Paulmichl, and F. Lang. Epinephrine activates outward rectifying K channel in Mardin-Darby canine kidney cells. *Pflügers Arch.* **408**:584–591 (1987).
14. Murata, M., F. Gusovsky, T. Yasumoto, and J. W. Daly. Selective stimulation of Ca^{2+} flux in cells by maitotoxin. *Eur. J. Pharmacol.* **227**:43–49 (1992).
15. Merritt, J. E., W. P. Armstrong, C. D. Benham, T. J. Hallam, R. Jacob, A. Jaxa-Chamiec, B. K. Leigh, S. A. McCarthy, K. E. Moores, and T. J. Rink. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.* **271**:515–522 (1990).
16. Krautwurst, D., J. Hescheler, D. Arndts, W. Löesel, R. Hammer, and G. Schultz. Novel potent inhibitor of receptor-activated nonselective cation currents in HL-60 cells. *Mol. Pharmacol.* **43**:655–659 (1993).
17. Schwarz, G., G. Droogmans, and B. Nilius. Multiple effects of SK&F 96365 on ionic currents and intracellular calcium in human endothelial cells. *Cell Calcium*, in press.

Send reprint requests to: Paul Dietl, Physiologisches Institut der Universität Innsbruck, Fritz-Pregl Str. 3, A-6020 Innsbruck, Austria.