Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2590–2594, April 1992
Neurobiology

Mobilization of dantrolene-sensitive intracellular calcium pools is involved in the cytotoxicity induced by quisqualate and *N*-methyl-D-aspartate but not by 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate and kainate in cultured cerebral cortical neurons

(neurotoxicity/neuroprotection)

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Communicated by Hans H. Ussing, December 11, 1991 (received for review August 21, 1991)

ABSTRACT **By using primary cultures of cerebral cortical neurons, it has been demonstrated that the antihyperthermia drug dantrolene protects against cytotoxicity induced by the excitatory amino acids quisqualate (QA) and *N*-methyl-D-aspartate (NMDA), whereas no effect was observed on cell damage mediated by kainate (KA) or 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate (AMPA). In parallel it was shown that KA and AMPA increased the concentration of intracellular free calcium ($[Ca^{2+}]_i$) mainly by influx, whereas the increase in $[Ca^{2+}]_i$ stimulated by NMDA and QA predominantly was caused by release of Ca^{2+} from intracellular stores, which for NMDA seemed to be mediated at least partly by Ca^{2+} influx. In accordance with the effects on cytotoxicity, dantrolene blocked the increase in $[Ca^{2+}]_i$ elicited by QA and NMDA leaving the increase induced by KA and AMPA unaffected. The finding that 2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionate, which regarding toxicity is a selective KA antagonist, only reduced the KA-stimulated increase in $[Ca^{2+}]_i$ by 30% may suggest that the elevation of $[Ca^{2+}]_i$ is not the only element in KA-induced cytotoxicity. On the other hand, the present study underlines the importance of Ca^{2+} for cytotoxicity induced by some excitatory amino acids (glutamate, NMDA, and QA) and supports the current proposal that multiple mechanisms are operating, even concerning calcium homeostasis. Because excitatory amino acid-induced cytotoxicity is thought to be involved in neuropathological conditions such as ischemia, it is possible that dantrolene might be of therapeutic interest.**

The neurotoxicity of glutamate and related excitatory amino acids (EAAs), which is thought to be involved in brain damage associated with pathological conditions such as hypoxia and ischemia (1, 2), is phenomenologically well described (3–8). The mechanisms by which this toxicity is exerted are, however, not completely understood, although there is ample evidence that changes in the cellular calcium homeostasis play a prominent role. Exposure to EAAs is always associated with elevated intracellular calcium concentrations, which may be the result of an increased influx and/or release of Ca^{2+} from intracellular stores. At present, the relative contribution to the observed increase in intracellular free calcium ($[Ca^{2+}]_i$) of influx and intracellular release is unknown. It seems that this ratio depends on the cell type (e.g., ref. 9) as well as on the type of the EAA inducing the cell damage (e.g., activation of a metabotropic response versus an ionotropic response). It is also not clear to what extent such elevations of $[Ca^{2+}]_i$ are involved in the etiology of EAA-induced cell damage. Therefore, it was important in the same model system to investigate the role of influx versus intracellular release for the increase in $[Ca^{2+}]_i$ induced by the individual EAAs also with a view to elucidate the significance of these routes to the cytotoxicity mechanisms.

Dantrolene, which is used in the clinic in symptomatic treatment of conditions such as malignant hyperthermia, neuroleptic malignant syndrome, and certain types of muscle spasticity (10), has been reported to inhibit Ca^{2+} release from sarcoplasmic reticulum in skeletal muscle and from unidentified Ca^{2+} stores in other cell types (for references, see ref. 10). In cultured cerebral cortical neurons dantrolene does not affect Ca^{2+} influx by voltage-gated Ca^{2+} channels (11). Also in cultured cerebellar granule cells, dantrolene has been shown to prevent the glutamate-induced increase in $[Ca^{2+}]_i$ (12). By employing dantrolene, it was subsequently found that the glutamate-induced increase in $[Ca^{2+}]_i$ in cerebral cortical neurons may be dissected into three pools; (*i*) a dantrolene-sensitive pool being independent of external calcium; (*ii*) a dantrolene-sensitive pool that is dependent on external calcium, and (*iii*) finally, a pool that is dependent on the presence of external calcium but is not sensitive to dantrolene (11). Since dantrolene even in the presence of external calcium has been found to completely protect against glutamate-induced cell death in analogous cultures of neurons (11), it may be concluded that release of calcium from intracellular stores triggered by either Ca^{2+} influx or by other second messengers may be centrally placed in the mechanism(s) responsible for glutamate-related cytotoxicity. On the other hand, the results suggested that Ca^{2+} influx may not be the primary event (11). Since there is increasing evidence to suggest that different mechanisms are employed in the cytotoxicity exerted by the various EAAs (13, 14), we decided to study the possible effects of dantrolene on changes in intracellular calcium homeostasis and cytotoxicity induced by EAAs other than glutamate. Thus, the purpose of the present study was to elucidate the possible diversity in the regulation of calcium homeostasis pertinent to the cytotoxicity induced by the various EAAs. A better understanding of these processes may lead to the discovery of neuroprotective

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Abbreviations: AMOA, 2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionate; AMPA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate; APV, D-2-amino-5-phosphonovalerate; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; EAA, excitatory amino acid; KA, kainate; NMDA, *N*-methyl-D-aspartate; QA, quisqualate; LDH, lactate dehydrogenase; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

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agents interfering with intracellular events that are secondary to activation of the different subtypes of EAA receptors.

MATERIALS AND METHODS

Materials. Pregnant mice (gestational day 15) were obtained from the animal quarters at the Panum Institute, University of Copenhagen. Plastic tissue culture dishes were purchased from NUNC A/S (Roskilde, Denmark), and fetal calf serum was from Sera-Lab (Crawley Down, Sussex, U.K.). Cytosine arabinoside, poly(L-lysine), trypsin, trypsin inhibitor, DNase, amino acids, vitamins, dantrolene, A23187, and verapamil were obtained from Sigma; enzymes and coenzymes were from Boehringer Mannheim. EAAs and their antagonists were obtained from Tocris Neuramin (Essex, U.K.) and 2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionate (AMOA) was synthesized and generously supplied by P. Krogsgaard-Larsen (PharmaBiotec Res. Ctr., Dept. of Org. Chem., The Royal Danish School for Pharmacy, Copenhagen). Pluronic F-127 and the acetoxymethyl ester of fluo-3 were obtained from Molecular Probes.

Cell Cultures. Cerebral cortical neurons were cultured essentially as described by Dichter (15), Hertz *et al.* (16), and Frandsen and Schousboe (5). Astrocytic proliferation was curtailed by addition of the mitotic inhibitor cytosine arabinoside (20 μM) after 48 h of culture (15, 17). The neurons were routinely cultured for 9 days before experiments were performed (for further details, see refs. 18 and 19).

Determination of Cytotoxicity. The compounds in question were added to the culture medium of neurons cultured for 9 days, and immediately before and at the end of the exposure period (9 h), samples were taken for determination of lactate dehydrogenase (LDH) activity as described by Frandsen and Schousboe (4) and Koh and Choi (20). Results obtained using the LDH-leakage viability test is compatible with data using conventional viability staining such as trypan blue (4). Concentrations of EAAs were chosen to obtain similar levels of cell damage. Sampling and measurements of LDH activity were performed as described (4, 6). Dantrolene was in all experiments used at a concentration of 30 μM, since this was the minimal concentration (tested over a concentration range of 1–250 μM) at which protection against toxicity induced by glutamate (11), *N*-methyl-D-aspartate (NMDA), or quisqualate (QA) (data not shown) was obtained and since the drug had some toxic actions when used at higher concentrations (data not shown).

Measurement of $[Ca^{2+}]_i$. All experiments were carried out using neurons cultured for 9 days. Fluo-3 (acetoxymethyl ester) loading and fluorescence measurements were performed according to the procedure of Wahl *et al.* (21) and Frandsen and Schousboe (11). To allow calculations of $[Ca^{2+}]_i$, cells were incubated with the ionophore A23187 [10 μM in Hepes-buffered saline (135 mM NaCl/5 mM KCl/0.62 mM $MgSO_4$/1.8 mM $CaCl_2$/10 mM Hepes/6 mM glucose, pH 7.4)] allowing sufficient influx of Ca^{2+} to attain the saturation level of binding with the intracellularly trapped fluo-3 ligand (F_{max}). Subsequently, the fluorescence was quenched with 2 mM $CuCl_2$ dissolved in 0.9% NaCl/10 μM A23187 to obtain the minimum fluorescence signal (F_{min}). Fluorescence was blanked against unloaded cells. The observed relative fluorescence values for the cells were used in the following equation to calculate $[Ca^{2+}]_i$: $K_d(F - F_{min})/(F_{max} - F)$, where the K_d is 450 nM and F is the observed fluorescence, which increases upon binding of Ca^{2+} without shifts in excitation or emission wavelengths (22). The neurons were exposed to the compounds in Hepes-buffered saline. The minimum concentration of dantrolene needed to block intracellular release of Ca^{2+} induced by glutamate (10 μM), NMDA (300 μM), or QA (300 μM) was 30 μM (tested over a concentration range from 1 to 250 μM).

RESULTS

In the presence of 1.8 mM extracellular Ca^{2+}, all EAAs tested increased $[Ca^{2+}]_i$ 8- to 14-fold over the basal level of 73 ± 2 nM (Table 1). Table 1 also shows the pharmacological profile of the EAA-mediated increase in $[Ca^{2+}]_i$. The glutamate-mediated increase could be slightly, but significantly, reduced by exposure of the cells to either 100 μM D-(−)-2-amino-5-phosphonovalerate (APV) or 25 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and totally blocked if a mixture of APV and CNQX was included in the incubation with glutamate. The NMDA-stimulated calcium response was unaffected by CNQX but was reduced 80% by APV. The residual 20% could be blocked by simultaneous addition of verapamil (5 μM) but not by CNQX (data not shown). The increase in $[Ca^{2+}]_i$ mediated by QA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate (AMPA), or kainate (KA) was blocked by CNQX, whereas APV did not have any effect. AMOA reduced the increase in $[Ca^{2+}]_i$ stimulated by KA by 30% but had no effect on those stimulated by any of the other EAAs. Verapamil significantly attenuated the responses to glutamate, AMPA, or KA whereas it had no effect on the increase in $[Ca^{2+}]_i$ stimulated by NMDA (in the absence of APV) or QA (Table 2).

When no calcium was added to the medium, the responses to all the EAAs were reduced (Fig. 1). The increase in $[Ca^{2+}]_i$ elicited by glutamate, NMDA, or QA was still significant although reduced to ≈40% of the responses obtained in the presence of 1.8 mM Ca^{2+}. On the contrary, exposure of the neurons to KA and AMPA under these conditions only led to small and statistically insignificant elevations of $[Ca^{2+}]_i$. Effects of exposure of the neurons to dantrolene in the presence of the EAAs are also shown in Fig. 1. In the presence of external $CaCl_2$, the AMPA-stimulated response was not affected by dantrolene and that of KA was only slightly diminished. In contrast, dantrolene completely prevented the QA-stimulated increase in $[Ca^{2+}]_i$ and greatly reduced the responses to NMDA and glutamate. Under conditions where $CaCl_2$ was omitted from the incubation medium, dantrolene could block the latter responses completely, whereas no effect on the small increases in $[Ca^{2+}]_i$ elicited by KA and AMPA was seen. To investigate whether the dantrolene-sensitive calcium pools might be involved in cytotoxicity induced by EAAs other than glutamate, the effect of dantrolene on the EAA-induced cytotoxicity was also investigated. Fig. 2 shows that dantrolene in the presence of 1.8 mM Ca^{2+} protected completely against cytotox-

Table 1. Pharmacology of EAA-induced increase in $[Ca^{2+}]_i$ with $[Ca^{2+}]_{ex}$ at 1.8 mM

	$[Ca^{2+}]_i$, nM				
Addition	Control	APV	CNQX	APV + CNQX	AMOA
None	73 ± 2	81 ± 7	77 ± 4	83 ± 3	113 ± 25
Glutamate	1070 ± 13	860 ± 10*	931 ± 6	75 ± 4*	994 ± 32
NMDA	890 ± 12	150 ± 5*	861 ± 9	ND	877 ± 36
QA	600 ± 7	621 ± 5	109 ± 8*	ND	571 ± 22
AMPA	573 ± 6	578 ± 7	71 ± 5*	ND	565 ± 18
KA	695 ± 9	703 ± 4	90 ± 7*	ND	445 ± 29*

Experiments were performed with cerebral cortical neurons cultured for 9 days. After the loading procedure, fluorescence was measured four times with 90-s intervals before test compounds were added in 50 μl of Hepes-buffered saline. The following concentrations of EAAs and their antagonists were used: glutamate, 10 μM; NMDA, QA, AMPA, and KA, 300 μM; APV, 100 μM; CNQX, 25 μM. Results are mean ± SEM of 36–48 experiments, except for AMOA where 16 experiments were performed. Asterisks indicate statistically significant differences from the appropriate control values using Kruskal–Wallis test for nonparametric analyses ($P < 0.001$). ND, not determined.

Table 2. Effect of verapamil (5 μM) on EAA-induced increase in $[Ca^{2+}]_i$ in cerebral cortical neurons with $[Ca^{2+}]_i$ at 1.8 mM

Addition	$[Ca^{2+}]_i$, nM	
	Control	Verapamil
None	71 ± 4	87 ± 7
Glutamate	1039 ± 15	896 ± 12*
NMDA	890 ± 12	840 ± 9
QA	603 ± 9	600 ± 7
AMPA	581 ± 6	370 ± 5*
KA	600 ± 11	400 ± 6*

For details, see Table 1. Values are the mean ± SEM of 30 experiments. Asterisks indicate statistically significant differences from the appropriate control values using Kruskal–Wallis test for nonparametric analyses ($P < 0.001$).

icity induced by NMDA or QA, whereas no effect was observed on cell damage induced by KA or AMPA. The dependency on external Ca^{2+} of the EAA-induced cytotoxicity was also studied. As shown in Table 3, only the cell damage induced by QA was unaffected by the omission of $CaCl_2$ from the incubation medium whereas the toxicity of KA, AMPA, NMDA, and glutamate was significantly attenuated under these conditions. If, however, the extracellular $[Ca^{2+}]$ was increased to 10 mM, the cytotoxicity of KA or AMPA was exacerbated whereas that of NMDA, QA, or glutamate was unaffected, the latter being already approximately maximal in the presence of 1.8 mM Ca^{2+}.

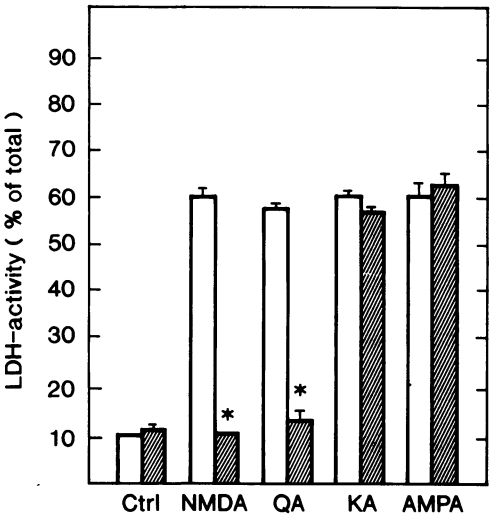

FIG. 2. Effects of dantrolene (30 μM) on the toxicity elicited by EAAs in the presence of culture medium containing 1.8 mM Ca^{2+}. Neurons were cultured for 9 days before exposure (9 h) to the compounds in question (NMDA, 300 μM; QA, 300 μM; KA, 300 μM; AMPA, 100 μM). Cell damage was evaluated by measuring the leakage of LDH to the culture medium (4, 6). Results are averages of nine experiments (mean ± SEM, shown by vertical lines). Asterisks indicate statistically significant differences using Kruskal–Wallis test for nonparametric analyses ($P < 0.001$). Bars are as in Fig. 1.

DISCUSSION

The finding that the increase in $[Ca^{2+}]_i$ elicited by all EAAs was diminished by removal of $CaCl_2$ from the incubation medium indicates that at least a part of this increase is related to stimulation by the EAAs of Ca^{2+} influx. This is in agreement with previous studies of $^{45}Ca^{2+}$ influx that showed that this influx could be stimulated by each of the EAA agonists (23). The dependency on extracellular Ca^{2+} was most pronounced for AMPA and KA, whereas the increase in $[Ca^{2+}]_i$ elicited by glutamate (11), QA, and NMDA was only partly dependent on external Ca^{2+}. This suggests that KA and AMPA increase $[Ca^{2+}]_i$ primarily by Ca^{2+} influx. The finding that the KA- and AMPA-induced increase in $[Ca^{2+}]_i$ could be partly blocked by verapamil indicates that voltage-dependent Ca^{2+} channels may be involved. This is consistent with a recent report by Weiss *et al.* (24) that nifedipine is able to counteract the neurotoxic action of KA in cultured cerebral cortical neurons. The partial independency on external Ca^{2+} of the glutamate- (11), NMDA-, and QA-stimulated increase in $[Ca^{2+}]_i$ suggests that these EAAs are able to mediate a mobilization of Ca^{2+} from intracellular stores. This is in agreement with previous findings in different types of neurons (12, 25). It is also compatible with the present finding that dantrolene, which prevents release of Ca^{2+} from intracellular stores (12, 26), could partly block the increase in

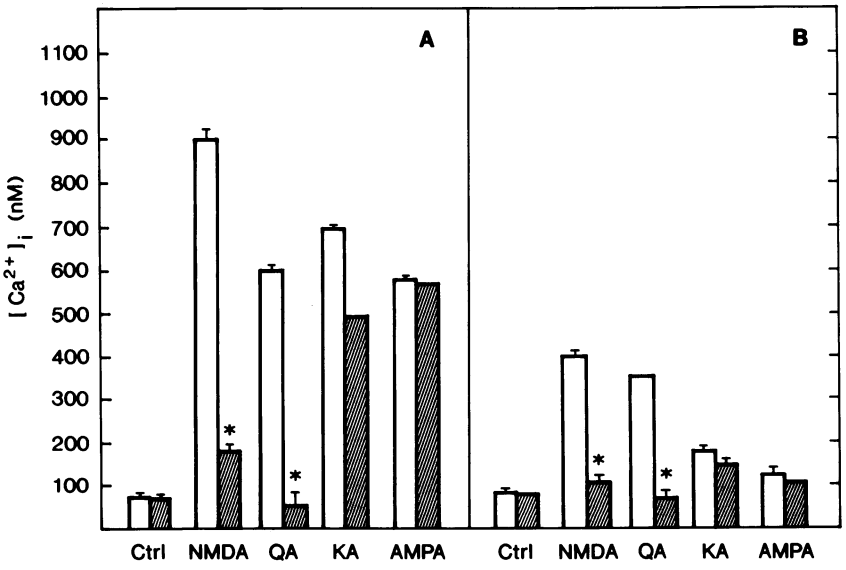

FIG. 1. Effects of dantrolene (30 μM) on EAA-stimulated increase in $[Ca^{2+}]_i$ in 9-day-old cerebral cortical neurons in the presence (*A*) or absence (*B*) of 1.8 mM Ca^{2+} added to the incubation medium. Results are from 72 to 144 experiments (mean ± SEM, indicated by vertical lines). Asterisks indicate statistically significant differences using Kruskal–Wallis test for nonparametric analyses ($P < 0.001$). Open bars show results obtained with the respective EAAs alone, and hatched bars represent the corresponding results from incubations with the respective EAAs plus dantrolene. Ctrl, control.

Table 3. Dependency on extracellular Ca^{2+} of EAA toxicity in cerebral cortical neurons

Condition	LDH activity, % of total		
	0	1.8 mM	10 mM
Control	13 ± 3	11 ± 11	16 ± 4
Glutamate	53 ± 3*	76 ± 2	80 ± 4
NMDA	34 ± 2*	60 ± 3	63 ± 2
KA	37 ± 5*	61 ± 2	77 ± 5*
QA	63 ± 6	58 ± 2	62 ± 5
AMPA	30 ± 5*	59 ± 3	75 ± 3*

Neurons were cultured for 9 days before exposure for 9 h to EAAs in the absence of added extracellular $CaCl_2$ (nominally 0 mM) or in the presence of 1.8 or 10 mM $CaCl_2$. Results are the mean ± SEM of nine experiments. Asterisks indicate statistically significant differences from the results obtained in the presence of 1.8 mM Ca^{2+} using Kruskal–Wallis test for nonparametric analyses ($P < 0.001$). For further details, see Table 1.

$[Ca^{2+}]_i$ evoked by glutamate and NMDA and completely block that elicited by QA in the presence of extracellular calcium. On the other hand, no effect of dantrolene was observed on the KA- or AMPA-stimulated changes in calcium homeostasis, which is consistent with the hypothesis (see above) that these EAAs primarily stimulate Ca^{2+} influx. This clearly shows that the different subtypes of glutamate receptors elicit changes in $[Ca^{2+}]_i$ by different mechanisms.

The pharmacological profiles of the EAA-stimulated increase in $[Ca^{2+}]_i$ are in agreement with previous findings (21, 27). The glutamate response showed a mixed EAA-receptor subtype profile since it could only be completely blocked in the presence of both the selective NMDA antagonist (28) APV and the selective non-NMDA antagonist (29) CNQX and was only partly reduced by either APV or CNQX alone. The NMDA-stimulated increase in $[Ca^{2+}]_i$ was exclusively attenuated by APV although not completely blocked. This residual APV-insensitive increase in $[Ca^{2+}]_i$ is, however, unlikely to be associated with non-NMDA receptors, since it consistently could not be abolished by CNQX but by verapamil, which blocks voltage-sensitive Ca^{2+} channels (30). The Ca^{2+} responses induced by AMPA, QA, or KA could be attenuated by the non-NMDA antagonist CNQX, although only the AMPA and the QA responses were completely blocked by CNQX. The CNQX-resistent QA response seems in contrast to the similar APV-resistant NMDA response not to be mediated by verapamil-sensitive Ca^{2+} channels. On the other hand, the increases in $[Ca^{2+}]_i$ seen after stimulation with either KA or AMPA are likely to be partly due to opening of such voltage-sensitive Ca^{2+} channels since the addition of verapamil plus the EAA in question reduced the respective responses significantly. The finding that CNQX still completely abolished the KA- and AMPA-induced responses may indicate that the function of these voltage-gated channels may be overall controlled by CNQX-sensitive structures. The conclusion that the KA- and AMPA-mediated increases in $[Ca^{2+}]_i$ were due to stimulation of Ca^{2+} influx and not to liberation of Ca^{2+} from intracellular stores is further substantiated by the finding that dantrolene, which prevents release of Ca^{2+} from such stores (for review, see ref. 10), could not prevent these increases in $[Ca^{2+}]_i$. On the other hand, since dantrolene partly blocked the glutamate (11), NMDA, and QA responses in the presence of $CaCl_2$ and totally blocked these responses in the absence of external Ca^{2+}, it is clear that these EAAs affect not only Ca^{2+} influx but also liberation of Ca^{2+} from intracellular stores. For glutamate and QA, this is in agreement with the previous demonstrations that these EAAs stimulate inositol phosphate metabolism in neurons (31). It was somewhat surprising that also the NMDA response was dantrolene sensitive, but this might indicate that the Ca^{2+} influx mediated by NMDA receptors may trigger release from intracellular stores, as was demonstrated for glutamate (11). This conclusion is in keeping with the present finding that NMDA toxicity was diminished under conditions with no external Ca^{2+}. The finding that dantrolene in addition to the neuroprotective properties against toxicity induced by glutamate (11) also protected against QA and NMDA cytotoxicity underlines the importance of Ca^{2+} release from internal stores in the mechanisms involved in the cytotoxicity of certain, but not all, EAAs.

Presently, no definite conclusion regarding the identity of the dantrolene-sensitive Ca^{2+} stores can be drawn. From the present study and a previous study by Frandsen and Schousboe (11), it is known that this antihyperthermia drug affects an internal Ca^{2+} store from which release occurs without a need for initial influx of Ca^{2+}. Since QA also stimulates release from such a pool, this may be an inositol trisphosphate-sensitive store. Furthermore, dantrolene inhibits release from a structure from which Ca^{2+} release is dependent on the presence of external free calcium (i.e., most likely representing a pool from which release is triggered by Ca^{2+} influx). This influx occurs supposedly through EAA-gated channels, since dantrolene does not affect Ca^{2+} influx by voltage-gated channels (11).

In summary, it can be concluded that Ca^{2+} plays a central role in the mechanisms for cytotoxicity induced by all EAAs and that these mechanisms with regard to the exact route of increasing $[Ca^{2+}]_i$ may be grouped in three categories: (*i*) a mechanism for which influx triggers Ca^{2+} release from dantrolene-sensitive internal stores (glutamate and NMDA); (*ii*) a dantrolene-sensitive process that is totally independent of Ca^{2+} influx (QA), and (*iii*) a mechanism that is insensitive to dantrolene and totally dependent on influx (KA and AMPA). The present finding that AMOA, which with respect to toxicity is a selective KA antagonist (7), reduced the increase in $[Ca^{2+}]_i$ stimulated by KA by only 30% may indicate that even though Ca^{2+} responses may be involved in KA-dependent cytotoxicity, it does not seem to be the causative signal. The initiating signal is at present not characterized, but it may be related to production of cGMP since AMOA has been shown to block increases in the intracellular concentration of cGMP stimulated by KA (13). Because the neuroprotective effect of dantrolene is due to interference with processes distally to the EAA-receptor activation, it is possible that some of the normal functions mediated by EAA receptors may be operative in the presence of this drug. It is accordingly likely that dantrolene therapy in combination with partial EAA receptor blockage may have less severe side effects than therapeutic strategies exclusively based on EAA-receptor antagonists.

The excellent technical assistance of Miss Charlotte Fredsøe Andersen is cordially acknowledged. The work was supported by grants from The NOVO Foundation, The Lundbeck Foundation, Director Ib Henriksen's Foundation, the Danish Biotechnology Programme (1991–1995), and the EEC Bridge Program (BIOT-0183-C).

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