Monocytic cells secrete factors that evoke excitatory synaptic activity in rat hippocampal cultures

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Abstract

Microglia- and macrophage-induced neuronal death may underlie a number of neurodegenerative diseases. The effects of factors secreted by monocytic cells were studied on glutamatergic synaptic transmission between cultured rat hippocampal neurons. Conditioned media from differentiated human U937 cells was collected after 24 h and applied to neurons (0.5%–30% dilution). Unactivated U937 cells spontaneously released factors that when applied to neuronal cultures evoked bursts of action potentials and elicited neuronal death ($29 \pm 4\%$). Conditioned media collected from U937 cells evoked intracellular calcium ($[Ca^{2+}]_i$) spiking (0.5%–2% dilution) and at higher concentrations evoked sustained increases in intracellular calcium (3%–30% dilution), as measured by indo-1-based photometry in single neurons. Activation of the U937 cells with zymosin A (500 μg/ml) enhanced the potency of the conditioned media to increase intraneuronal $[Ca^{2+}]_i$ as indicated by a leftward shift in the concentration–response curve. Selective antagonists to voltage-gated Na^+ and Ca^{2+} channels and NMDA-gated channels (tetrodotoxin, nimodipine, and ($\pm$)-2-amino-5-phosphonopentanoic acid, respectively) blocked the calcium transients elicited by unactivated and zymosin -A-treated conditioned media. This pharmacologic profile is consistent with U937-released factors that excite the synaptic network that forms between cultured hippocampal neurons. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Monocyte; $[Ca^{2+}]_i$; Glutamate; Excitatory synaptic transmission

1. Introduction

Central nervous system mononuclear phagocytes are thought to participate in the neuronal death underlying a number of neurodegenerative diseases. Microglia, macrophages and multi-nucleated macrophage-like cells apparently direct specialized cytotoxic processes against the nervous system contributing to the neuronal death that accompanies acquired immunodeficiency syndrome (AIDS) dementia complex, multiple sclerosis, Parkinson's disease and Alzheimer's disease (Nakajima and Kohsaka, 1993). The mechanism underlying glia-mediated toxicity has yet to be established. Activation of mononuclear phagocytes is thought to generate a variety of soluble substances such as cytokines, reactive oxygen intermediates, nitric oxide, tissue plasminogen activator, and the NMDA receptor ligands quinolinate and glutamate that cause neurotoxicity to nearby neurons (Giulian and Corpuz, 1993; Chao et al., 1995, 1996; Heyes et al., 1996).

NMDA receptor antagonists can attenuate the neurotoxicity caused by many of the substances released by mononuclear phagocytes (Chao et al., 1996). Excessive stimulation of NMDA receptors produces a rise in the neuronal intracellular calcium ($[Ca^{2+}]_i$) and subsequent neuronal injury/death. Increases in $[Ca^{2+}]_i$ are thought to represent a final common pathway for many neurological insults that induce neurodegenerative diseases (Randall and Thayer, 1992; Choi, 1994). Factors that produce NMDA receptor-mediated toxicity can act via direct receptor activation or by stimulation of endogenous glutamate release. To explore the possibility that mononuclear phagocyte activation might produce neurotoxicity by indirect mechanisms we examined soluble factors secreted by a monocytic cell line for effects on neuronal survival and glutamatergic synaptic activity. U937 cell-secreted factors excited the synaptic network that forms between cultured hippocampal neurons and caused significant neuronal death.

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2. Materials and methods

2.1. Cell culture

The human promonocytic cell line U937 was grown as a suspension culture in RPMI 1640 media supplemented with 5% fetal bovine serum, penicillin/streptomycin (100 units/ml and 0.1 mg/ml, respectively) and 20 mM HEPES (pH 7.45) at 37°C in a humidified, 95% air, 5% CO_2 atmosphere as described previously (Sundstrom and Nilsson, 1976).

To prepare conditioned media, U937 cells were plated onto growth flasks that had been coated with collagen (5 $\mu g/cm^2$) at a density of 20,000 cells /ml and differentiated with 5 nM phorbol myristate acetate in RPMI 1640 growth media. After 2 days in culture, the media was changed to phorbol myristate acetate-containing RPMI 1640 growth media that lacked glutamine, aspartate, and glutamate. The U937 cells were morphologically differentiated after the 3-day exposure to phorbol myristate acetate. The round U937 cells grown in suspension differentiated into flat cells displaying pseudopodia and adhering to the growth flask and to other cells. After the 3-day differentiation, phorbol myristate acetate was removed and conditioned media was collected 24 h later. Conditioned media was centrifuged at 1000 rpm for 10 min and aliquots were stored at 4°C (up to 14 days) or −20°C (up to 4 months) without loss of activity.

Fetal rat hippocampal neurons were isolated as described previously (Shen et al., 1996), and plated at a density of 50,000 cells/well onto 25-mm round coverglasses that had been coated with poly-D-lysine (0.1 mg/ml) and washed with H_2O.

2.2. Toxicity

For neurotoxicity assays, hippocampal neurons were plated on micro-etched coverslips at a density of 50,000 cells/well as described above. Approximately 100 neurons were counted on each coverslip. Coverslips were then treated with conditioned media (10% v/v) by removing a portion of the culture media and replacing it with the conditioned media. Forty-eight hours later, the same fields of cells were recounted by an investigator blind to treatment. A neuron was considered healthy if it was phase bright, had rounded somata and extended long fine processes. Cell death was determined by comparing the number of viable neurons before and after treatment.

2.3. Statistical methods

Throughout this study, ''n'' designates the number of different flasks of conditioned media. In most cases, each flask of conditioned media was tested using more than two neurons (for microfluorimetry) or two coverslips (for toxicity). Data are expressed as means ± S.E.M. Paired two-tailed Student's t-test was used to determine significance.

2.4. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was determined using a previously described dual emission microfluorimeter (Werth and Thayer, 1994) to monitor the calcium-sensitive fluorescent chelator, indo-1 (Grynkiewicz et al., 1985). Cells were loaded with indo-1 by incubation with 2 μM indo-1 acetoxymethyl ester for 45 min at 37°C, in HEPES buffered Hanks' salt solution (HHSS), pH 7.45, containing 0.5% bovine serum albumin. HHSS was composed of the following (in mM): HEPES, 20; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.4; KH_2PO_4, 0.4; Na_2HPO_4, 0.3; $NaHCO_3$, 3.0; glucose, 5.6. Loaded cells were mounted in a flow-through chamber for viewing (Thayer et al., 1987). The superfusion chamber was mounted on an inverted microscope and cells were superfused with 0.5%–30% excitatory amino acid deficient RPMI growth media in HHSS containing 10 μM glycine at a rate of 1.0–1.5 ml /min for 15 min prior to starting an experiment. The $[Ca^{2+}]_i$ baseline was not affected when the perfusion solution was switched from HHSS to excitatory amino acid deficient RPMI growth media in HHSS.

2.5. Electrophysiology

Whole cell recordings were obtained from cultured neurons with pipettes (3–5 MΩ resistance) pulled from borosilicate glass. Pipettes were filled with a solution containing (in mM) 125 K gluconate, 10 KCl, 10 HEPES, 5 Mg ATP, 10 EGTA. The osmolarity of the internal recording solution was adjusted to 315 mosM/kg with sucrose and the pH was adjusted to 7.2 with KOH. Whole cell recordings were established in an extracellular recording solution containing (in mM) 140 NaCl, 5 KCl, 0.9 $MgCl_2$, 5 glucose, 0.001 glycine, and 10 HEPES, pH 7.4 with NaOH. The extracellular solution was adjusted to 325 mosM/kg with sucrose. Whole cell currents were recorded with an Axopatch 200A patch-clamp amplifier and the BASIC-FASTLAB interface system (Indec systems).

3. Results

Hippocampal neurons were grown in culture for 12–15 days by which time they had formed extensive synaptic connections. For viability assays, the neurons were grown on coverslips with micro-etched grids that enabled identified cells to be counted before and 48 h after treatment with media conditioned by the U937 monocytic cell line. Conditioned media was collected from phorbol myristate acetate-differentiated U937 cells 24 h after growth in excitatory amino acid-deficient RPMI growth media as described in Section 2. Neurons were treated with conditioned media by replacing 10% (300 μl) of the hippocampal culture media with U937-conditioned media. As shown in Fig. 1, the addition of excitatory amino acid-deficient
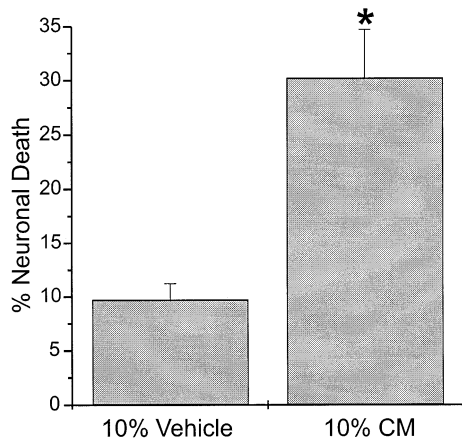
Fig. 1. U937 cells spontaneously secrete soluble factors that are neurotoxic. Hippocampal neuronal death was determined by cell counts after 48-h exposure to 10% vehicle (excitatory amino acid deficient RPMI growth media) or 10% U937-conditioned media (CM), as described in Section 2. Conditioned media from three separate flasks was examined for neurotoxicity. For each treatment, two coverslips were averaged. Data are displayed as percent neuronal death (mean $\pm$ S.E.M.; $n = 3$) above that observed in the media exchange control ($36 \pm 1\%$). * Signifies $p < 0.05$.

RPMI (vehicle) did not significantly affect neuronal viability. Treatment with U937-conditioned media (10%) for 48 h produced $29 \pm 4\%$ neuronal death, a significant increase relative to vehicle-treated cultures ($p < 0.05$). Glutamate (100 μM) elicited $62 \pm 8\%$ neuronal death in this assay. Thus, consistent with prior reports (Colton and Gilbert, 1987; Giulian and Corpuz, 1993; Giulian et al., 1992), macrophage-like cells secrete factors that kill neurons.

An elevation in $[Ca^{2+}]_i$ is an early and essential event known to initiate neurotoxicity (Randall and Thayer, 1992; Choi, 1994). We explored the possibility that U937-secreted factors might evoke an increase in $[Ca^{2+}]_i$ in hippocampal neurons. U937-conditioned media was superfused onto hippocampal neurons while $[Ca^{2+}]_i$ was monitored with indo-1-based photometry. As shown in Fig. 2A, conditioned media evoked a rapid and reversible increase in neuronal $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase was dependent on the concentration of conditioned media (Fig. 2C). Note that $[Ca^{2+}]_i$ transients evoked by low concentrations of conditioned media differed not only in amplitude but also in the shape of the response (Fig. 2B). Low concentrations of conditioned media (0.5%–2% v/v) evoked a series of $[Ca^{2+}]_i$ spikes (Fig. 2B), whereas higher concentrations elicited a large $[Ca^{2+}]_i$ transient possibly resulting from a fusion of the spikes (Fig. 2A; inset).

The secretion of cytotoxic substances from macrophages can be activated by immunostimulants such as zymosin A (Colton and Gilbert, 1987; Giulian et al., 1992). Conditioned media collected from zymosin A (500 μg/ml)-treated U937 cells was more potent at increasing neuronal $[Ca^{2+}]_i$ than media collected from unactivated monocytic cells as indicated by the leftward shift in the concentra-
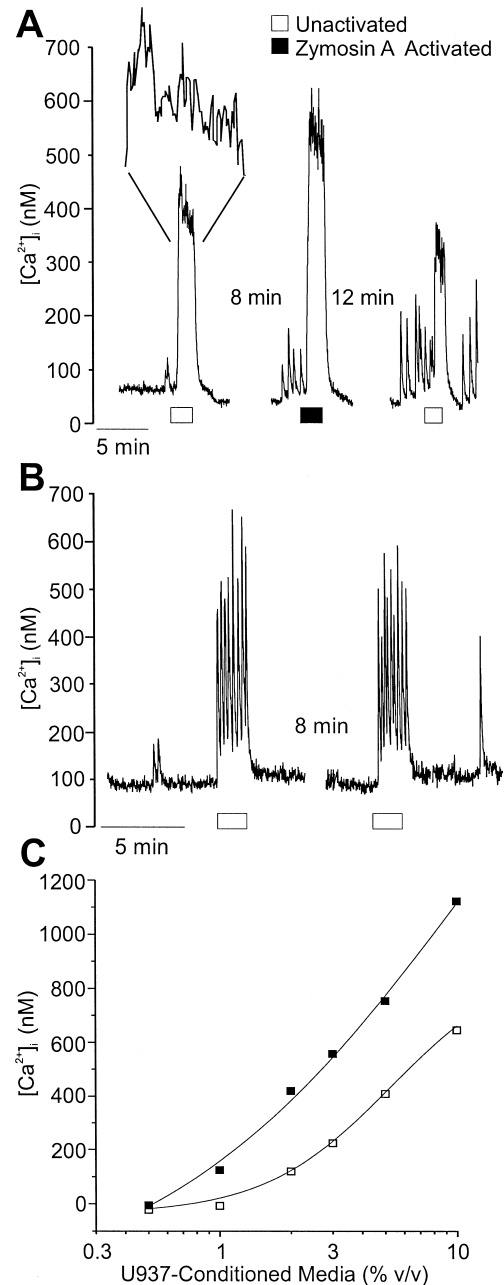

Fig. 2. U937-conditioned media increases neuronal $[Ca^{2+}]_i$. (A) A 2-min superfusion of 3% unactivated U937-conditioned media onto a single hippocampal neuron, as indicated by the horizontal bar, elicited an increase in $[Ca^{2+}]_i$. Superfusion of 3% conditioned media from zymosin A-activated (500 μg/ml) U937 cells onto the same hippocampal neuron elicited a larger $[Ca^{2+}]_i$ transient. The inset displays, on an expanded scale, the fused oscillations in $[Ca^{2+}]_i$. (B) Superfusion of a lower concentration (2%) of conditioned media onto a single neuron evoked $[Ca^{2+}]_i$ spiking. (C) Increases in $[Ca^{2+}]_i$ are dependent on the concentration of conditioned media. A single neuron was challenged with various concentrations of conditioned media (0.5%–10 %) collected from unactivated (open squares) or zymosin A-treated (solid squares) U937 cells. Each concentration of conditioned media was superfused onto a neuron twice. The order of application was varied and the $[Ca^{2+}]_i$ amplitudes averaged.

tion–response curve (Fig. 2A and C). When 3% conditioned media from zymosin A treated- and untreated U937 cells was tested on the same neuron, media collected from zymosin A-activated U937 cells increased the $[Ca^{2+}]_i$ to 436 ± 42 nM ($n = 11$; 48 neurons), significantly greater ($p < 0.05$, paired t-test) than the 342 ± 53 nM increase elicited by media collected from unactivated cells.

U937-conditioned media that had been frozen at $-80°C$, then thawed, produced comparable increases in neuronal $[Ca^{2+}]_i$ (data not shown). Likewise, heat-treated conditioned media (10 min, 95°C) also produced an increase in

neuronal $[Ca^{2+}]_i$ (data not shown). Thus, the secreted factor studied here is stable and not a short-lived compound such as a free radical.

The $[Ca^{2+}]_i$ spiking activity elicited by low concentrations of U937-conditioned media is reminiscent of the $[Ca^{2+}]_i$ spiking activity that results from an aberrant pattern of glutamatergic synaptic activity observed in in vitro models of epilepsy (McLeod et al., 1998). To test this idea we used pharmacologic agents to selectively block ion channels that participate in excitatory synaptic activity. Synaptic activity requires voltage-gated Na^+ channels that
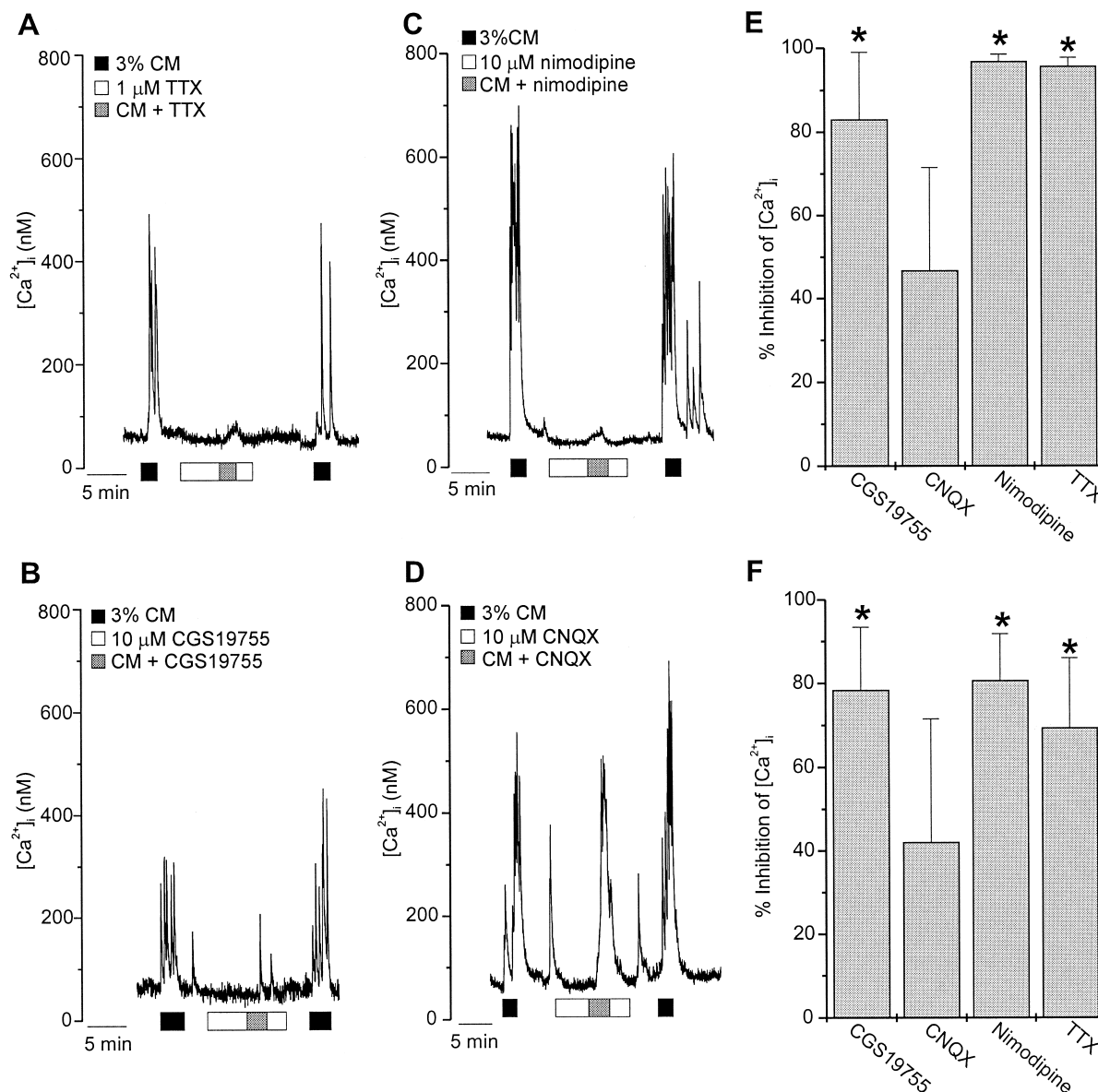

Fig. 3. Pharmacologic characterization of U937-conditioned media-elicited $[Ca^{2+}]_i$ transients. Unactivated U937 cell-conditioned media (CM) was superfused onto single hippocampal neurons to evoke an increase in $[Ca^{2+}]_i$ as indicated by horizontal bars. After the $[Ca^{2+}]_i$ had returned to baseline the neuron was superfused with (A) 1 μM TTX, (B) 10 μM CGS19755, (C) 10 μM nimodipine, or (D) 10 μM CNQX for 5 min. Conditioned media was then superfused in the maintained presence of the pharmacological agent for 2 min. After the $[Ca^{2+}]_i$ had returned to baseline, a second application of conditioned media alone was applied. Summaries of the pharmacological characterization of (E) unactivated U937-conditioned media and (F) zymosin A-activated U937-conditioned media are shown. Each pharmacological agent was tested with conditioned media derived from three different flasks and with two neurons per conditioned media preparation. Data are presented as mean $\pm$ S.E.M. ($n = 3$). * Signifies $p < 0.01$.

can be blocked by tetrodotoxin (TTX). TTX (1 μM) completely blocked the increase in $[Ca^{2+}]_i$ evoked by conditioned media (Fig. 3A; $n = 3$; six neurons; $p < 0.01$). Epileptic bursting, particularly that known to produce $[Ca^{2+}]_i$ spiking and neurotoxicity, requires the activation of NMDA receptors (Traub et al., 1994; McLeod et al., 1998). The competitive NMDA receptor antagonist $(\pm)$-2-amino-5-phosphonopentanoic acid (CGS19755) (10 μM) completely blocked the increase in $[Ca^{2+}]_i$ evoked by conditioned media (Fig. 3B; $n = 3$; six neurons; $p < 0.01$). We have found that L-type voltage-gated Ca^{2+} channels also participate in synaptically mediated neurotoxicity and $[Ca^{2+}]_i$ spiking in hippocampal cultures. Consistent with a role for L-type Ca^{2+} channels in the conditioned media-evoked $[Ca^{2+}]_i$ increase, 10 μM nimodipine also completely blocked the response (Fig. 3C; $n = 3$; six neurons; $p < 0.01$). The role of non-NMDA-gated glutamate receptors in the $[Ca^{2+}]_i$ spiking and neurotoxicity in vitro is less clear and other model systems have yielded variable sensitivity to the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). CNQX (10 μM) inhibited the conditioned media-evoked response by $53 \pm 25\%$, in good agreement with the partial inhibition observed in our previous studies of synaptically mediated neurotoxicity (McLeod et al., 1998) (Fig. 3D; $n = 3$; six neurons).

We also characterized the $[Ca^{2+}]_i$ spiking activity evoked by low concentrations of conditioned media collected from zymosin A-activated U937 cells. As with the conditioned media from unactivated cells, TTX, nimodipine and CGS19755 all completely blocked the zymosin A-conditioned media-evoked response ($p < 0.01$; $n = 3$; six neurons), and CNQX inhibited the response by $42 \pm 30\%$. As summarized in Fig. 3E and F, these pharmacological studies indicate that the factors secreted by mono-

cytic cells are able to excite the network of hippocampal neurons that forms in culture such that an aberrant pattern of glutamatergic synaptic activity is produced.

We next performed whole-cell current-clamp experiments in which membrane potential was monitored during application of condition media. Treatment of a quiescent cell with 3% conditioned media evoked intense bursts of action potentials (Fig. 4; $n = 4$). We have shown previously that each burst of action potentials will produce a $[Ca^{2+}]_i$ spike (McLeod et al., 1998). Clearly, conditioned media excites neurons producing aberrant patterns of electrical activity.

4. Discussion

We have shown that the monocytic cell line U937 secretes stable factors that excite neurons and produce neuronal death. Previous studies demonstrated that monocytic cells could be induced to release substances that kill neurons by activating NMDA receptors (Giulian et al., 1990; Piani et al., 1991; Lipton, 1992b; Espey et al., 1997). We show here that these factors do not necessarily need to activate glutamate receptors directly, but rather, may act via an indirect mechanism in which the synaptic network that forms between cultured hippocampal neurons is over-activated leading to excitotoxicity. We suggest this possibility based on the pharmacology of the response elicited by U937-conditioned media, the characteristic waveform of the $[Ca^{2+}]_i$ transient as well as the bursting pattern of action potentials that we have shown previously to produce neuronal death by a synaptic mechanism (McLeod et al., 1998).

U937-conditioned media elicited $[Ca^{2+}]_i$ spiking that was reminiscent of the $[Ca^{2+}]_i$ spiking activity that results from an aberrant pattern of glutamatergic synaptic activity (McLeod et al., 1998). Use of receptor antagonists and channel blockers indicated that the NMDA receptor and the L-type Ca^{2+} channel were essential for $[Ca^{2+}]_i$ spiking. This finding is consistent with other reports that found that inhibition of the NMDA receptor protected from the neurotoxic agent(s) secreted by mononuclear phagocytes (Giulian et al., 1992; Lipton, 1992a; Chao et al., 1996). L-type voltage-gated Ca^{2+} channels have been implicated in the neurotoxicity induced by the human immunodeficiency virus type-1 (HIV-1) envelope protein, gp120 (Dreyer et al., 1990; Lo et al., 1992), which is thought to act by inducing the release of neurotoxins from microglia (Giulian et al., 1990; Lipton, 1992a). The role non-NMDA-receptors play in the elicited $[Ca^{2+}]_i$ spiking was less clear. However, consistent with our previous work (Traub et al., 1994; McLeod et al., 1998), the role of non-NMDA receptors may be to relieve the voltage-dependent Mg^{2+} block of the NMDA receptor. Thus, the contribution of these receptors will vary depending on the resting potential of the postsynaptic cell. These observa-
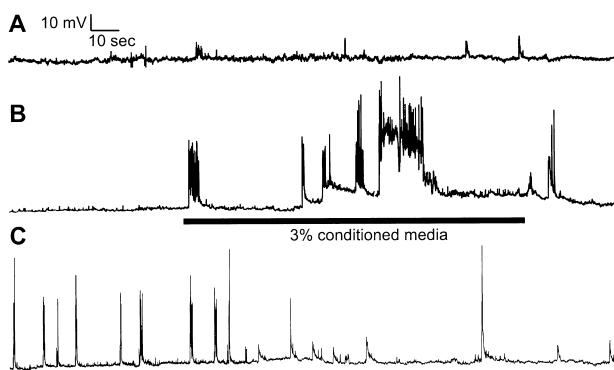

Fig. 4. Conditioned media evokes bursts of electrical activity. Whole cell patch clamp was used to record electrical activity in hippocampal neurons. (A) Under control conditions, action potentials were infrequent. (B) Superfusion with 3%-conditioned media (120 s) evoked repetitive bursts of action potentials. (C) The frequency and duration of action potential bursts decreased during the wash period. Recording is representative of four experiments.

tions suggest that U937-released factors act, in part, by an indirect mechanism to excite the synaptic network leading to excitotoxic death.

Because the secreted factors induce an aberrant pattern of synaptic activity, there are a number of potential sites for the induction of this activity as well as targets to which neuroprotective agents could be directed. Modulation of membrane excitability by K^+ channel inhibition or a reduction in inhibitory synaptic tone will produce synaptic bursting in in vitro models of epilepsy (McBain, 1994; Avoli et al., 1996). Alternatively, modulation of NMDA receptor sensitivity by kinases, zinc and co-agonists might also be plausible targets (Christine and Choi, 1990; Wang and Salter, 1994). The oscillatory nature of the $[Ca^{2+}]_i$ spiking, with at times a complete recovery between spikes, suggests that if the conditioned media acts directly its effects were amplified considerably by other mechanisms. In addition, we have applied various concentrations of glutamate to the culture and recorded abrupt and sustained increases in $[Ca^{2+}]_i$ that increased in amplitude in a concentration dependent manner; in no instance was oscillatory $[Ca^{2+}]_i$ spiking observed (Hegg and Thayer, unpublished observations). Finally, in another report we found that modulation of glutamate release was effective in protecting hippocampal cultures from this type of synaptically mediated cell death (Shen et al., 1996).

Zymosin A activation of U937 cells increased the potency of the conditioned media, although activation was not essential for secretion of factors. Unlike previous observations, U937 cells did not require an activator such as fixed *Staphylococcus aureus,* zymosin A particles, HIV-1, or the HIV-1 envelope protein gp120 to secrete neurotoxic factors (Giulian et al., 1990, 1992, 1993,1996). This may result in part from an increased sensitivity of the $[Ca^{2+}]_i$ response relative to cell viability assays. The sensitivity of our bioassay required that the media used to culture U937 cells be formulated to enable single cell $[Ca^{2+}]_i$ measurements. The excitatory amino acids glutamine, glutamate and aspartate were removed from the U937 cell growth media. Under these conditions, unactivated U937 cells release a factor that is able to cause neuronal death. It is possible that this media induced the cells to release toxic factors. In previous studies in which the mononuclear phagocyte growth media contained excitatory amino acids, unactivated U937 cells (Giulian et al., 1990, 1996) and other mononuclear phagocytes (Giulian et al., 1992, 1993) did not secrete toxins that killed neurons. It is not clear whether the excitatory amino acids in the monocyte growth media affected the neuronal viability in these previously published studies.

Monocytic cells release factors that induce a neurotoxic pattern of glutamatergic synaptic activity. We have developed a sensitive bioassay that may prove useful in the identification and study of these factors. The possibility that monocyte-induced neuronal death might occur via a synaptic mechanism suggests that study of the mechanism

of this process, as well as efforts to prevent it, should not focus exclusively on the NMDA receptor, but rather, encompass the entire synaptic network.

Acknowledgements

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