# Mediation of Neuronal Apoptosis by Enhancement of Outward Potassium Current

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Apoptosis of mouse neocortical neurons induced by serum deprivation or by staurosporine was associated with an early enhancement of delayed rectifier ($I_K$) current and loss of total intracellular $K^+$. This $I_K$ augmentation was not seen in neurons undergoing excitotoxic necrosis or in older neurons resistant to staurosporine-induced apoptosis. Attenuating outward $K^+$ current with tetraethylammonium or elevated extracellular $K^+$, but not blockers of $Ca^{2+}$, $Cl^-$, or other $K^+$ channels, reduced apoptosis, even if associated increases in intracellular $Ca^{2+}$ concentration were prevented. Furthermore, exposure to the $K^+$ ionophore valinomycin or the $K^+$-channel opener cromakalim induced apoptosis. Enhanced $K^+$ efflux may mediate certain forms of neuronal apoptosis.

Neurons undergo apoptosis during normal development and in certain disease states (*1*). Elevated extracellular $K^+$ interdicts this death (*2*, *3*), an effect attributed to increasing $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels, thus increasing intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) toward a set point that is optimal for survival (*3*). The antiapoptotic effect of high $K^+$ concentration can be attenuated by removing extracellular $Ca^{2+}$ or adding $Ca^{2+}$-channel blockers (*3*, *4*), and neuronal survival can be enhanced by opening $Ca^{2+}$ channels with dihydropyridine agonists (*4*, *5*) or inhibiting intracellular $Ca^{2+}$ sequestration with thapsigargin (*6*). However, although these data support an antiapoptotic effect of increasing $[Ca^{2+}]_i$, they do not exclude other possibilities. Furthermore, increases in $[Ca^{2+}]_i$ can induce apoptosis under some conditions (*7*), and, in the absence of nerve growth factor, a high concentration of $K^+$ promoted survival of sympathetic neurons without an increase in $[Ca^{2+}]_i$ (*8*).

To test the idea that high extracellular $K^+$ might also attenuate neuronal apoptosis by reducing $K^+$ efflux, we first examined whether neurons undergoing apoptosis exhibited an up-regulation of outward $K^+$ currents. Cultured mouse cortical neurons in mixed (neurons and glia) or near-pure neuronal cultures (*9*, *10*) were studied with whole-cell recording between 7 and 12 days in vitro (DIV) (*11*). A persistent outward current, consistent with the delayed rectifier $I_K$ (Fig. 1A), and a transient outward current, consistent with $I_A$ (Fig. 1B) (*12*), were the two major voltage-gated $K^+$ currents observed. $I_K$ was reduced by increasing extracellular $K^+$ (Fig. 1C) and exhibited slow kinetics, outward rectification, and sensitivity to tetraethylammonium (TEA) (Fig. 1A) (*13*). $I_A$ exhibited rapid activation and inactivation as well as sensitivity to 4-aminopyridine (4AP) (Fig. 1B). A small inward rectifier current activated with hyperpolarization and a small outward current consistent with the ATP-sensitive $K^+$ current ($K_{ATP}$) was also observed. Other major $K^+$ currents, the M-current ($I_M$) and the $Ca^{2+}$-dependent, high-conductance $K^+$ current (BK current), were not detected.

In seven to nine DIV neurons in neuronal cultures, the steady-state current of $I_K$ activated by voltage jump from −70 to +40 mV was 506 ± 46 pA ($n$ = 25 cells, mean ± SEM). Six hours after serum withdrawal, $I_K$ at +40 mV was increased by 61% and the maximal $K^+$ conductance more than doubled without change in voltage sensitivity or kinetics (Fig. 2, A, C, and D; Table 1). Holding current at −70 mV shifted from −18 ± 6 pA at baseline to −2 ± 4 pA after 5 hours of serum deprivation ($P < 0.05$; $n$ = 44 and 23, respectively), indicative of membrane hyperpolarization. Cell capacitance gradually decreased, consistent with progressive cell body shrinkage (Table 1), and total intracellular $K^+$ dropped by 7 ± 3% and 13 ± 4%, respectively, after 5 and 9 hours in serum-free medium (both different from baseline at $P < 0.05$; $n$ = 10) (*14*). The enhanced $I_K$ remained sensitive to block by TEA (Fig. 2B); 5 mM TEA completely prevented loss of intracellular $K^+$.

No change in $I_K$ was seen in neurons exposed to a sham wash (Fig. 2C). The enhancement in $I_K$ induced by serum deprivation was not blocked by cycloheximide (1 μg/ml) (Table 1), suggesting that the enhancement did not require new protein synthesis.

Neuronal apoptosis induced in mixed cultures (DIV 10 to 12) by 0.1 μM staurosporine was also associated with an enhancement in $I_K$. Despite an initial nonsignificant trend toward reduced $I_K$ after 30 min, after 9 to 11 hours $I_K$ was increased by 90% and maximum $K^+$ conductance was doubled (Table 1). In contrast, $I_K$ did not change in neurons that underwent *N*-methyl-D-aspartate (NMDA)–induced excitotoxic necrosis (Table 1), although by 11 hours neurons exhibited substantial cell swelling. Furthermore, exposure to staurosporine did not alter $I_K$ in older neurons (DIV 17; $n$ = 5), which survive such exposure (*15*).

Serum deprivation and staurosporine exposure also altered the transient current $I_A$, although with opposite effects. At times of

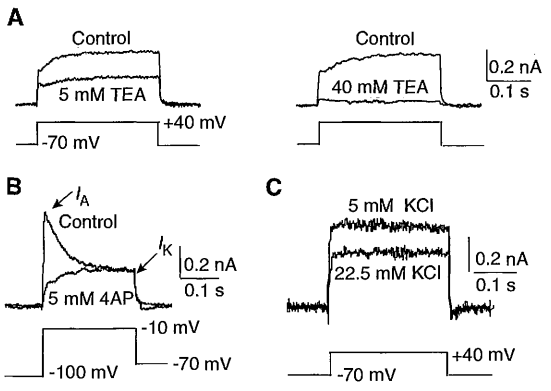


**Fig. 1.** Voltage-gated $K^+$ currents in cultured cortical neurons. (**A**) Normal $I_K$ recorded from neurons in mixed neuronal glial cultures. Current was activated by stepping from a holding potential of −70 to +40 mV for 300 to 600 ms, with leak current subtraction. The noninactivating outward current was dose dependently blocked by 1 to 40 mM bath-applied TEA (effects shown are 5 and 40 mM). (**B**) 4AP selectively blocked a transient outward $K^+$ current, $I_A$. Currents were activated by a voltage step from −100 to −10 mV. The initial transient outward current was blocked by 5 mM 4AP, consistent with $I_A$. The 4AP resistant current was blocked by TEA, consistent with $I_K$ as shown in (A). (**C**) $I_K$ was sensitive to extracellular $K^+$ concentration. When $K^+$ was increased from 5 to 22.5 mM, the same voltage step activated a smaller outward current, similar to the effect of 5 mM TEA. The inhibitory effect of medium with high $K^+$ concentration is expected from a reduced driving force for $K^+$ efflux, predicted by the Nernst equation.

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**Table 1.** Effects of apoptotic and necrotic insults on $I_K$. $I_K$, steady-state current activated by voltage step from −70 to +40 mV; $G_{max}$, maximum conductance; $V_{threshold}$, voltage threshold for $I_K$ activation; $C_M$, membrane capacitance; $V_{2y1}$, voltage for $I_K$ half activation; $\tau_{opening}$, time constant for $I_K$ opening at +40 mV measured in the presence of 5 mM 4AP by single exponential curve fitting. These parameters were examined before (control) and at plateau levels after insults: 11 hours in staurosporine (0.1 μM), 6 hours in serum deprivation, and 11 hours in 15 μM NMDA (plus 10 μM glycine; DIV 10 to 12 mixed cultures). Cycloheximide concentration was 1 μg/ml. Serum deprivation and staurosporine exposure were performed in pure neuron cultures and mixed cultures, respectively (*9*, *10*).

| | $I_K$ (pA) | $G_{max}$ (nS) | $C_M$ (pF) | $I_K$ density (pA/pF) | $V_{threshold}$ (mV) | $V_{1/2}$ (mV) | $\tau_{opening}$ (ms) |
|---|---|---|---|---|---|---|---|
| Control | 506 ± 46 | 4.1 ± 0.5 | 31.6 ± 2.4 | 25.2 ± 6.7 | −33.3 ± 3.1 | 2.6 ± 1.1 | 21.7 ± 3.6 |
| Serum deprivation | 815 ± 61* | 9.7 ± 0.2* | 24.9 ± 2.1* | 47.0 ± 4.9* | −34.5 ± 2.5 | 1.6 ± 0.9 | 28.8 ± 3.3 |
| Control | 512 ± 81 | 4.6 ± 0.6 | 32.0 ± 2.8 | 15.2 ± 2.6 | −33.0 ± 3.2 | 2.5 ± 1.0 | 22.1 ± 3.5 |
| Staurosporine | 969 ± 145* | 9.8 ± 2.6* | 23.9 ± 2.0* | 31.8 ± 7.2* | −38.1 ± 1.0* | −2.1 ± 0.6* | 25.9 ± 4.0 |
| Control | 361 ± 59 | — | — | — | — | — | — |
| Serum deprivation + cycloheximide | 760 ± 149* | — | — | — | — | — | — |
| Control | 597 ± 97 | — | — | — | — | — | — |
| NMDA | 565 ± 151 | — | — | — | — | — | — |

*Significant difference ($P < 0.05$) from respective control by $t$ -test; $n = 5$ to 25 (most $n \geq 10$).

maximal change, serum deprivation (6 hours) increased $I_A$ by 28% ($P < 0.05$; $n = 20$), whereas staurosporine (0.1 μM; 9 hours) decreased $I_A$ by 75% ($P < 0.05$; $n = 8$). Exposure to NMDA did not alter $I_A$. Because activity of voltage-gated $Ca^{2+}$ channels may affect apoptosis, we monitored high-voltage–activated (HVA) $Ca^{2+}$ currents during serum deprivation and found no significant change (232 ± 35 pA in control cells, 192 ± 33 and 147 ± 51 pA after 6 and 9 hours; $P = 0.43$ and 0.22, respectively; $n = 5$ per condition). Tested after 5 to 9 hours in 0.1 μM staurosporine, the $I_M$ and BK currents remained undetectable; the inward rectifier and $K_{ATP}$ current were not altered.

The selective enhancement of $I_K$ induced by either serum deprivation or staurosporine exposure, as well as the increase in $I_A$ induced by the former, occurred before development of neuronal apoptotic death (*16*), consistent with a critical early role. In support of this idea, adding 1 to 5 mM TEA or increasing extracellular $K^+$ from 5 to 25 mM reduced both forms of apoptosis (Fig. 3, A and B). In contrast, neither the TEA analog tetramethylammonium (5 mM; inactive on $I_K$) nor 4AP (5 mM; antagonist for the slowly inactivating $K^+$ current $I_D$ as well as $I_A$) was effective (Fig. 3B). Other antagonists that targeted $K_{ATP}$ (tolbutamide; 100 to 500 μM) or the SK channel (apamin; 1 μM) also lacked protective effect. TEA may inhibit $Cl^-$ currents in cortical neurons (*17*); however, no neuroprotection was observed with the $Cl^-$-channel antagonist anthracene-9-carboxylic acid (500 μM) (data not shown).

We considered the possibility that the protective effect of TEA was mediated by an increase in $[Ca^{2+}]_i$. Neuronal $[Ca^{2+}]_i$ measured with fura-2 (*18*) was about 100 nM at rest and increased to a plateau value of about 200 nM after 1 to 2 hours of exposure to 5 mM TEA or 25 mM $K^+$. Gadolinium (2 to 10 μM), which completely blocked the HVA $Ca^{2+}$ current (Fig. 4A), kept $[Ca^{2+}]_i$ at resting level during 2 to 16 hours of exposure to TEA or 25 mM $K^+$ (Fig. 4B); neither gadolinium (Fig. 4C) nor the L-type $Ca^{2+}$-channel antagonist nifedipine (5 μM; Fig. 4D) blocked the anti-apoptotic effects. Neither gadolinium nor nifedipine showed neuroprotection when applied alone (Fig. 4, B and C).

We considered the possibility that membrane depolarization might mediate the anti-apoptotic effects of TEA or high $K^+$ concentration. However, the $Na^+$-channel opener veratridine, which depolarized the membrane from control −51 ± 2 mV ($n = 45$) to −30 ± 2 mV ($n = 21$), similar to the membrane depolarization by 5 mM TEA (−34 ± 4 mV; $n = 15$), increased staurosporine-induced neuronal cell death (Fig. 3B).

In support of the hypothesis that increased $K^+$ efflux might be a primary step leading to apoptosis, application of the selective $K^+$ ionophore valinomycin (*19*) triggers apoptosis in thymocytes, lymphocytes, and tumor cells (*20*). Exposure to 20 nM valinomycin for 24 to 48 hours induced typical neuronal apoptosis in cortical cultures, characterized by chromatin condensation, cell body shrinkage, internucleosomal DNA fragmentation, and sensitivity to cycloheximide (1 μg/ml) or the caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (100 μM) (zVAD; Fig. 5). Furthermore, 24 to 48 hours of exposure to the $K^+$-channel opener cromakalim, which activates $K_{ATP}$ channels as well as $I_K$-like currents in mammalian central neurons (*21*), also induced typical neuronal apoptosis (Fig. 5).

In summary, four arguments suggest that a long-lasting enhancement of outward $K^+$

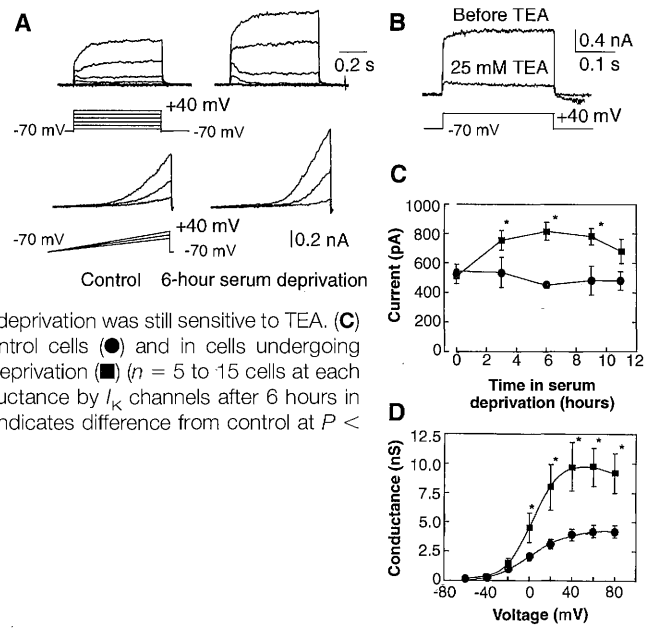


**Fig. 2.** Enhancement of $I_K$ by serum deprivation. (**A**) (Left) Recordings from DIV 9 neurons in pure neuronal cultures showing the *I-V* relationship of $I_K$ as revealed by rectangular and ramp voltage steps. (Right) After 6 hours of serum deprivation, the same voltage steps triggered much larger currents and outward rectification. (**B**) The $I_K$ enhanced by serum deprivation was still sensitive to TEA. (**C**) Change in $I_K$ with time in control cells (●) and in cells undergoing apoptosis induced by serum deprivation (■) ($n = 5$ to 15 cells at each point). (**D**) Increased $K^+$ conductance by $I_K$ channels after 6 hours in serum-free medium. Asterisk indicates difference from control at $P < 0.05$ by $t$ -test.

current is a key mediator of the forms of cortical neuronal apoptosis studied here. First, augmentation in $I_K$ and loss of neuronal cell $K^+$ occurred early in the course of neuronal apoptosis, well before the commitment point (*16*). The magnitude of this increase in $K^+$ current is comparable with that associated with mitogenesis (*22*) and proliferation (*23*) in several cell types (~0.5- to 3-fold increases of peak outward $K^+$ current). Second, this $I_K$ augmentation was specific to apoptosis but not triggered by necrotic insult or in older cells resistant to apoptosis. Third, blocking of this $I_K$ enhancement and cellular $K^+$ depletion by TEA or by increasing extracellular $K^+$ prevented apoptosis. Finally, increasing membrane $K^+$ permeability by adding either the ionophore valinomycin or the endogenous $K^+$-channel opener cromakalim sufficed to induce neuronal apoptosis.

In isolation, the third argument stated above is unsurprising, because both TEA and raising extracellular $K^+$ increase neuronal excitation and $[Ca^{2+}]_i$, and thus reduced apoptosis would be predicted by the $Ca^{2+}$ set-point hypothesis. However, neuroprotection was maintained even when associated increases in $[Ca^{2+}]_i$ were completely blocked. Some membrane-associated signaling proteins such as adenylyl cyclase may be voltage sensitive (*24*), but comparable membrane depolarization induced by veratridine was not neuroprotective.

Involvement of $K^+$ efflux in apoptosis has intuitive appeal, because loss of cell volume is a cardinal feature of apoptosis, and $K^+$ extrusion is a plausible mechanism to achieve this loss (*25*). The $I_A$ blocker 4AP recently has been reported to inhibit the shrinkage of human eosinophils undergoing cytokine deprivation-induced apo-

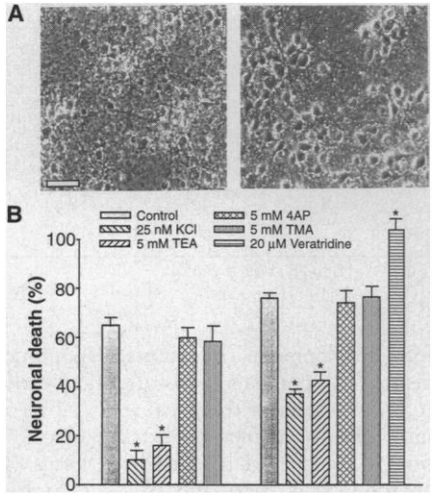


**Fig. 3.** Prevention of apoptosis by $I_K$ blocker TEA or by raising extracellular $K^+$ concentration. Pure neuronal culture (DIV 7 to 9) in 24-well plates was used for serum deprivation because the presence of glia prevents neuronal degeneration after serum deprivation. Mixed culture containing neurons and a glia bed (DIV 10 to 12) was used for exposure to staurosporine (0.1 μM). NMDA receptor antagonist 1 μM dizocilpine maleate {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801)} and non-NMDA receptor blocker 2,3-dihydro-6-nitro-7-sulfamoylbenzo(f)quinoxaline (5 μM) were added to block glutamate excitotoxicity in serum-deprivation experiments. Neuronal death was detected 24 and 48 hours after apoptotic insult. (**A**) (Left) Phase-contrast micrograph of a pure neuronal culture 48 hours after onset of serum deprivation, showing widespread neuronal apoptosis. (Right) Preservation of neurons in serum-free medium in the presence of 5 mM TEA. Bar = 50 μm. (**B**) Neuronal apoptosis, expressed as a fraction of the total number of neurons, induced by 48 hours of exposure to serum deprivation (left) or 0.1 μM staurosporine (right), either alone or in the presence of the indicated bath-applied drug (mean ± SEM; $n$ = 4 to 16 cultures per condition). Serum-deprivation–induced cell death was assessed by cell counts after staining with 0.4% trypan blue dye. For staurosporine experiments, cell death was measured by lactate dehydrogenase released into the medium (*30*). Cell deaths by both insults were assayed alternatively by two methods and similar results were obtained. Higher concentrations of TEA (20 mM) were toxic. Asterisk indicates significant difference from the control at $P < 0.05$ by $t$-test with Bonferroni correction for four comparisons.

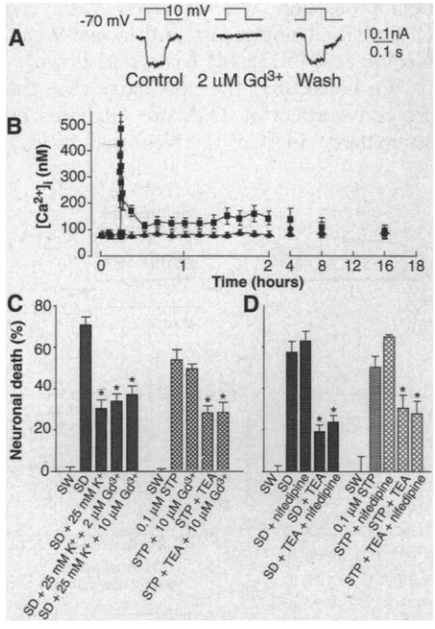


**Fig. 4.** Protective effects of TEA and 25 mM $K^+$ were not dependent on an increase in $[Ca^{2+}]_i$. (**A**) HVA $Ca^{2+}$ currents, activated by a voltage step from −70 to +10 mV, were blocked completely by bath-added 2 μM gadolinium ($Gd^{3+}$). The same results were obtained from three additional experiments. (**B**) Before break, bath application of 5 mM TEA (■) triggered an initial sharp increase in $[Ca^{2+}]_i$ as measured by fura-2 video microscopy, followed by relaxation to a lower plateau value (mean ± SD; $n > 30$ neurons for each time point). Results were similar in two additional experiments. TEA plus staurosporine produced a pattern of $[Ca^{2+}]_i$ increase similar to that produced by TEA alone; exposure to 0.1 μM staurosporine alone for 2 hours did not alter baseline neuronal $[Ca^{2+}]_i$ (data not shown). $Gd^{3+}$ (5 μM) completely blocked the TEA-induced increase in $[Ca^{2+}]_i$ in the presence of 0.1 μM staurosporine. After break, in the presence of TEA plus $Gd^{3+}$ (▲), $[Ca^{2+}]_i$ remained at resting levels for up to 16 hours (mean ± SD; $n \geq 200$ cells from four experiments). Similarly, 2 μM $Gd^{3+}$ completely prevented the 25 mM $K^+$-induced $[Ca^{2+}]_i$ increase (data not shown). ●, wash control. (**C**) Ability of 5 mM TEA or 25 mM $K^+$ to reduce neuronal apoptosis induced by serum deprivation (SD) (left) or staurosporine exposure (STP) (right) was not blocked by coapplication of 2 or 10 μM $Gd^{3+}$ ($n$ = 20 to 32 cultures per condition except $n$ = 4 for staurosporine plus $Gd^{3+}$ condition). SW, sham wash. (**D**) Neuroprotective effects of 5 mM TEA or 25 mM $K^+$ against apoptosis induced by either serum deprivation SD (left) or staurosporine STP (right) were not affected by coapplication of 5 μM nifedipine ($n$ = 12 for serum deprivation and $n$ = 4 for staurosporine experiments). Cell death was measured as described in Fig. 3. Asterisk indicates significant difference from control at $P < 0.05$ by $t$ -test with Bonferroni correction for two or three comparisons.

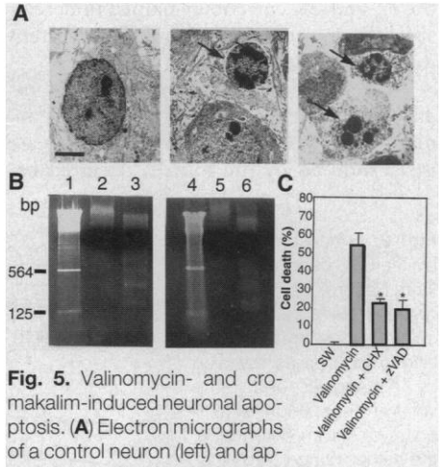


**Fig. 5.** Valinomycin- and cromakalim-induced neuronal apoptosis. (**A**) Electron micrographs of a control neuron (left) and apoptotic neurons (arrows) induced by 24 hours of exposure to the selective $K^+$ ionophore valinomycin (20 nM) (middle) or by the $K^+$-channel opener cromakalim (500 μM) (right). Bar = 3 μm. (**B**) DNA laddering on agarose gels after 24 hours of exposure to valinomycin (lane 3) or cromakalim (lane 6). The marker columns (lanes 1 and 4) show Hind III-digested λ DNA. Lanes 2 and 5, controls. (**C**) Valinomycin-induced neuronal death, assayed by staining with 0.4% trypan blue dye, was attenuated by addition of cycloheximide (1 μg/ml) (CHX; $n$ = 12) or 100 μM zVAD ($n$ = 12). Asterisk indicates significant difference from control at $P < 0.05$ by $t$ -test with Bonferroni correction for two comparisons. SW, sham wash.

ptosis (*26*). Additional study is needed to delineate the exact mechanisms by which activation of $I_K$ might promote neuronal apoptosis. One possible arena for linkage between these events is in cell cycle control, because $K^+$ channels and a decrease in intracellular $K^+$ have been implicated in initiation of mitosis (*27*), and apoptosis has been postulated to reflect an "abortive mitosis" (*28*). Furthermore, agents that reduce intracellular $K^+$ may activate caspases in macrophages or monocytes (*29*). We suggest that interventions directed at blocking excessive $K^+$ efflux, in particular by the noninactivating delayed rectifier $K^+$ channel, be explored as a strategy for attenuating neuronal apoptosis in disease states.

## REFERENCES AND NOTES


1. M. C. Raff *et al.*, *Science* **262**, 695 (1993); C. B. Thompson, *ibid.* **267**, 1456 (1995); D. W. Choi, *Curr. Opin. Neurobiol.* **6**, 667 (1996).
2. B. S. Scott and K. C. Fisher, *Exp. Neurol.* **27**, 16 (1970); M. R. Bennett and W. White, *Brain Res.* **173**, 549 (1979); A. Chalazonitis and G. D. Fischbach, *Dev. Biol.* **78**, 173 (1980); F. Collins, M. F. Schmidt, P. B. Guthrie, S. B. Kater, *J. Neurosci.* **11**, 2582 (1991).
3. V. Gallo, A. Kingsbury, R. Balázs, O. S. Jorgensen, *J. Neurosci.* **7**, 2203 (1987); J. L. Franklin and E. M. Johnson Jr., *Trends Neurosci.* **15**, 501 (1992); Y. Enokido and H. Hatanaka, *Neuroscience* **57**, 965 (1993); J-Y. Koh *et al.*, *Exp. Neurol.* **135**, 153 (1995); A. de Luca, M. Weller, A. Fontana, *J. Neurosci.* **16**, 4174 (1996).
4. T. Koike, D. P. Martin, E. M. Johnson Jr., *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6421 (1989); E. M. Johnson Jr., T. Koike, J. Franklin, *Exp. Neurol.* **115**, 163 (1992); J. L. Franklin, C. Sanz-Rodriguez, A. Juhasz, T. L. Deckwerth, E. M. Johnson Jr., *J. Neurosci.* **15**, 643 (1995).
5. C. Galli, *J. Neurosci.* **15**, 1172 (1995).
6. P. A. Lampe, E. B. Cornbrooks, A. Juhasz, E. M. Johnson Jr., J. L. Franklin, *J. Neurobiol.* **26**, 205 (1995).
7. N. Takei and Y. Endo, *Brain Res.* **652**, 65 (1994); D. J. McConkey and S. Orrenius, *J. Leukocyte Biol.* **59**, 775 (1996).
8. R. D. Murrell and A. M. Tolkovsky, *Eur. J. Neurosci.* **5**, 1261 (1993).
9. K. Rose, M. P. Goldberg, D. W. Choi, in *Methods in Toxicology*, C. A. Tyson and J. M. Frazier, Eds. (Academic Press, San Diego, CA, 1993), pp. 46–60.
10. J-Y. Koh, B. J. Gwag, D. Lobner, D. W. Choi, *Science* **268**, 573 (1995).
11. Whole-cell voltage clamp was performed on cortical cultures of DIV 7 to 17 in 35-mm culture dishes on the stage of an inverted microscope (Nikon, Japan) with an EPC-7 amplifier (List-Electronic, Germany). The extracellular solution contained 115 mM NaCl, 2.5 mM KCl, 2.0 mM $MnCl_2$, 10 mM Hepes, 0.1 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (BAPTA), 10 mM D-glucose, and 0.1 μM tetrodotoxin (TTX). The electrode solution contained 120 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 2.0 mM $Na_2ATP$, 1.0 mM BAPTA, and 10 mM Hepes. For HVA $Ca^{2+}$ current recording, the external solution contained 120 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 10 mM TEA, 0.0005 mM TTX, and 10 mM glucose. The electrode solution for HVA $Ca^{2+}$ currents contained 130 mM CsCl, 3 mM $MgCl_2$, 10 mM Hepes, 2.5 mM BAPTA, and 5 mM $Na_2ATP$. Experiments were performed at room temperature (21° to 22°C) and pH 7.3.
12. P. R. Adams and M. Galvan, *Adv. Neurol.* **44**, 137 (1986).
13. B. Hille, *J. Gen. Physiol.* **50**, 1287 (1967); E. Neher and H. D. Lux, *Pfluegers Arch.* **336**, 87 (1972).
14. For K measurement, pure neuronal cultures were washed with $K^+$-free solution, and cell membranes were disrupted with 0.1% Triton X-100; the extract from eight cultures was pooled for each $K^+$ measurement (performed with a $K^+$-sensitive electrode). Even 9 hours after the onset of serum deprivation, intracellular lactate dehydrogenase and total protein content remained normal, which is an argument against nonspecific membrane leakage or cell death.
15. J. W. McDonald, M. I. Behrens, C. Chung, T. Bhattacharyya, D. W. Choi, *Brain Res.*, in press.
16. Apoptosis was detected by DNA fragmentation (*10*) and cellular alterations. For electron microscopy, cultures were fixed and then embedded. Sections were cut and stained with lead citrate and uranyl acetate. Cells were examined and photographed with a JEOL 100 CS electron microscope. Tested by applying cycloheximide, phorbol 12-myristate 13-acetate, or BDNF, or re-adding serum, the commitment point for most neurons to die was ≥10 hours after apoptotic insults (M. I. Behrens, C. Csernansky, Dugan, D. W. Choi, unpublished data).
17. D. Y. Sanchez and A. L. Blatz, *J. Gen. Physiol.* **100**, 217 (1992).
18. For fura-2 fluorescence video microscopy, cultures of DIV 12 to 17 were exposed to 5 μM fura-2/AM plus 0.1% pluronic F-127 for 30 min at room temperature on the stage of a Nikon Diaphot inverted microscope. Fura-2 (excitation: 340, 380 nm; emission: 510 nm) ratio images were acquired with a charge-coupled device camera (Quantex) and digitized (256 × 512 pixels) by using a Metafluor 2.5 system (Universal Imaging, West Chester, PA). Background fluorescence was subtracted. Calibrated values were obtained in situ by determining $F_{min}$ (2 mM EGTA in $Ca^{2+}$ free-solution) and $F_{max}$ (10 μM ionomycin in 10 mM $Ca^{2+}$ solution). A $K_d$ of 225 nM was used in the ratio method formula.
19. B. C. Pressman, *Annu. Rev. Biochem.* **45**, 501 (1976).
20. N. L. Allbritton, C. R. Verret, R. C. Wolley, H. N. Eisen, *J. Exp. Med.* **167**, 514 (1988); D. M. Ojcius, A. Zychlinsky, L. M. Zheng, J. D.-E. Young, *Exp. Cell Res.* **197**, 43 (1991); C. L. P. Deckers, A. B. Lyons, K. Samuel, A. Sanderson, A. H. Maddy, *ibid.* **208**, 362 (1993); R. C. Duke, R. Z. Witter, P. B. Nash, J. D.-E. Young, D. M. Ojcius, *FASEB J.* **8**, 237 (1994).
21. D. M. T. Politi, S. Suzuki, M. A. Rogawski, *Eur. J. Pharmacol.* **168**, 7 (1989).
22. C. Deutsch, *Prog. Clin. Biol. Res.* **334**, 251 (1990); B. Rouzaire-Dubois and J-M. Dubois, *Cell Signal* **3**, 333 (1991).
23. S. C. Lee, D. E. Sabath, C. Deutsch, M. B. Prystowsky, *J. Cell Biol.* **102**, 1200 (1986); T. Konishi, *J. Physiol. (London)* **411**, 115 (1989).
24. R. Reddy *et al.*, *J. Biol. Chem.* **270**, 14340 (1995).
25. G. Barbiero, F. Duranti, G. Bonelli, J. S. Amenta, F. M. Baccino, *Exp. Cell Res.* **217**, 410 (1995).
26. F. Beauvais, L. Michel, L. Dubertret, *J. Leukocyte Biol.* **57**, 851 (1995).
27. C. D. Cone Jr. and C. M. Cone, *Science* **192**, 155 (1976); T. E. DeCoursey, K. G. Chandy, S. Gupta, M. D. Cahalan, *Nature* **307**, 465 (1984).
28. D. S. Ucker, *New Biol.* **3**, 103 (1991); G. Ferrari and L. A. Greene, *EMBO J.* **13**, 5922 (1994); N. Heintz, *Trends Biochem. Sci.* **18**, 157 (1993); L. L. Rubin, K. L. Philpott, S. F. Brooks, *Curr. Biol.* **3**, 391 (1993); G. Kroemer, P. Petit, N. Zamzami, J-L. Vayssière, B. Mignotte, *FASEB J.* **9**, 1277 (1995).
29. D. Perregaux and C. A. Gabel, *J. Biol. Chem.* **269**, 15195 (1994); I. Walev, K. Reske, M. Palmer, A. Valeva, S. Bhakdi, *EMBO J.* **14**, 1607 (1995).
30. J-Y. Koh and D. W. Choi, *J. Neurosci. Methods* **20**, 83 (1987).


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# Immunotherapy of Tumors with Autologous Tumor-Derived Heat Shock Protein Preparations


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**Immunotherapy of mice with preexisting cancers with heat shock protein preparations derived from autologous cancer resulted in retarded progression of the primary cancer, a reduced metastatic load, and prolongation of life-span. Treatment with heat shock protein preparations derived from cancers other than the autologous cancer did not provide significant protection. Spontaneous cancers (lung cancer and melanoma), chemically induced cancers (fibrosarcoma and colon carcinoma), and an ultraviolet radiation-induced spindle cell carcinoma were tested, and the results support the efficacy of autologous cancer–derived heat shock protein–peptide complexes in immunotherapy of cancers without the need to identify specific tumor antigenic epitopes.**


Heat shock proteins (HSPs) GP96, HSP90, and HSP70, when purified from cells, are associated with a broad range of peptides derived from that particular cell, such that the HSPs chaperone the antigenic repertoire of the cells from which they are purified (*1*). Immunization with HSP-peptide complexes, whether derived endogenously (*2–4*) or reconstituted in vitro (*5, 6*), elicits potent T cell responses against the chaperoned peptides and hence against the cells from which the HSPs are purified, as seen in studies with cancers (*1, 3, 7–9*), viruses (*4, 10–12*), model antigens (*5, 6, 13*), and minor histocompatibility antigens (*13*). We now examine the use of HSP-peptide complexes in the treatment of a variety of established cancers of spontaneous and experimental origin.

The 3LL (Lewis lung) carcinoma of C57BL/6 mice (*14, 15*) is a nonimmunogenic spontaneous cancer. It metastasizes naturally to the lungs if injected subcutaneously or in the footpad. HSP GP96 prepa-


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