remnant liver removal. Binding reactions were performed essentially as described (*3, 4*) with nuclear extracts from mouse liver cells after hepatectomy. For STAT binding, the probe used was a previously annealed high-performance liquid chromatography–purified double-stranded oligonucleotide from the sis-inducible factor binding element in the c-*fos* promoter (5′-GATCCTCCAGCATTTCCCGTAAATCCTCCAG-3′) (*22*) and end-labeled with [γ-^{32}P]adenosine triphosphate (ATP). Supershift experiments were performed by incubating 1 μl of primary antibody with the nuclear extracts in binding buffer for 1 to 2 hours at 4°C before addition of the labeled oligonucleotide. Antibody to STAT3 (anti-STAT3) and anti-STAT5 were from Santa Cruz Biotechnology.

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Neuroprotection by Aspirin and Sodium Salicylate Through Blockade of NF-κB Activation

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Aspirin (acetylsalicylic acid) is a commonly prescribed drug with a wide pharmacological spectrum. At concentrations compatible with amounts in plasma during chronic anti-inflammatory therapy, acetylsalicylic acid and its metabolite sodium salicylate were found to be protective against neurotoxicity elicited by the excitatory amino acid glutamate in rat primary neuronal cultures and hippocampal slices. The site of action of the drugs appeared to be downstream of glutamate receptors and to involve specific inhibition of glutamate-mediated induction of nuclear factor kappa B. These results may contribute to the emerging theme of anti-inflammatory drugs and neurodegeneration.

Glutamate is the most abundant excitatory neurotransmitter in the brain; however, under certain conditions, it may become a potent excitotoxin and contribute to neurodegeneration (*1*). On the other hand, an accumulation of clinical and experimental evidence suggests that neurodegeneration is often associated with inflammation (*2*). We tested the possibility that the anti-inflammatory drugs aspirin [acetylsalicylic acid (ASA)] and sodium salicylate (NaSal), because of their wide spectrum of pharmacological activities and multiple sites of action (*3*), may confer neuroprotective properties.

Several models of neurons in culture have been used to unravel the molecular events triggered by glutamate that lead to cell death as well as to develop pharmacological compounds able to counteract excitotoxicity. Here we used primary cultures of rat cerebellar granule cells, where a brief pulse of glutamate, through activation of the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor, induces cell death (*4*). ASA and NaSal were added to the culture medium 5 min before and during a 15-min application of 50 μM glutamate (*5*), a concentration that reduced cell survival by 70 to 80%. The range of concentrations for both drugs was correlated with the amounts in plasma (1 to 3 mM) for optimal anti-inflammatory effects in patients with rheumatic diseases (*3*). A concentration-dependent protection against glutamate-induced neurotoxicity was observed in the presence of both drugs (Fig. 1A). For ASA, the calculated median effective concentration (EC_{50}) was 1.7 mM, with maximal effect (83% protection) at 3 mM. The concentration of NaSal giving 50% protection was 5 mM, and maximal response (87% protection) was observed at 10 mM. Unlike salicylates, at concentrations compatible with the plasma levels during chronic drug treatment (1 to 20 μM) (*3*), the anti-inflammatory drug indomethacin was unable to prevent glutamate-induced cell death (*6*).

Neuroprotection was also evaluated in hippocampal slices of 8-day-old rat brain

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(7), a system that more closely represents in vivo conditions. In the hippocampal slices, most pyramidal neurons of CA1 and CA3 and granule cells of dentate gyrus (DG) became acutely necrotic, exhibiting swollen cytoplasm with large vacuoles, nuclear shrinkage, and focal clumping of chromatin (Fig. 1B). Application of ASA preserved hippocampal cell viability from the NMDA-mediated injury (Fig. 1, B and C). ASA did not modify cell viability at 1 mM, but at 3 mM it specifically produced significant neuroprotection in the CA3 region (Fig. 1C). Higher concentrations of ASA completely inhibited the NMDA effect in CA1 and DG as well as in CA3 cells (Fig. 1, B and C). Compared with primary cultures of rat cerebellar granule cells, 2 mM NaSal efficiently counteracted NMDA-mediated toxicity in hippocampal slices (Fig. 1C).

To dissect the molecular mechanisms by which salicylates preserved cell viability against excitoxicity, we tested whether these drugs diminished glutamate-mediated calcium entry (8). In rat cerebellar granule cells, application of glutamate in the absence of external Mg^{2+} caused a rapid increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a sustained plateau (Fig. 2A), principally because of the NMDA receptor subtype activation (9). ASA, applied at neuroprotective concentrations (1 to 3 mM), induced a very low and short-lasting $[Ca^{2+}]_i$ increase and did not modify glutamate-mediated calcium entry (Fig. 2B). Similar results were obtained with NaSal (9). Thus, it was likely that salicylates were acting on intracellular molecular targets further downstream of glutamate receptor activation, a property that makes them distinguishable from most neuroprotective drugs. It also appears that neuroprotection occurred independently of mechanisms controlling $[Ca^{2+}]_i$ homeostasis.

At plasma concentrations maintained during treatment of chronic inflammatory diseases, ASA and NaSal, but not indomethacin, inhibit the activation of nuclear factor kappa B (NF-κB)/Rel transcription factors in T and pre-B lymphocytes (10). The NF-κB/Rel family is implicated in controlling expression of several genes crucially involved in immune and inflammatory function (11). NF-κB/Rel proteins are present in primary neurons and in several brain areas (12). Administration of glutamate to primary cultures of rat cerebellar granule cells also results in up-regulation of NF-κB nuclear activity (13) and of the transcriptional complex AP-1 (14). Cells were exposed to 50 μM glutamate in the absence or presence of ASA (1 or 3 mM) and NaSal (3 or 10 mM), and nuclear extracts (15) were prepared 1 hour after stimulation. Both drugs

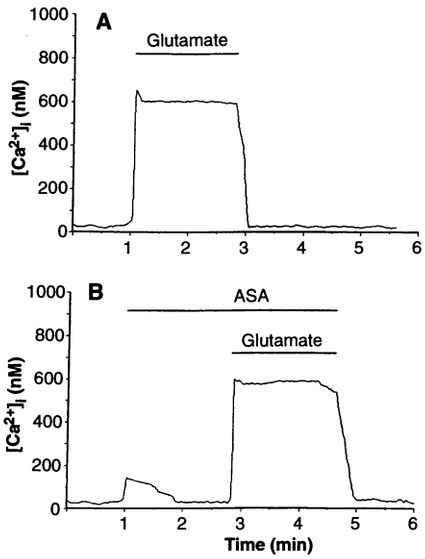

Fig. 2. Original recording showing the glutamate-induced $[Ca^{2+}]_i$ increase in rat cerebellar granule cells. (**A**) Effect of 50 μM glutamate (n = 95). (**B**) Effect of 50 μM glutamate in neurons pretreated with 3 mM ASA (n = 98). Traces are from representative cell recordings.

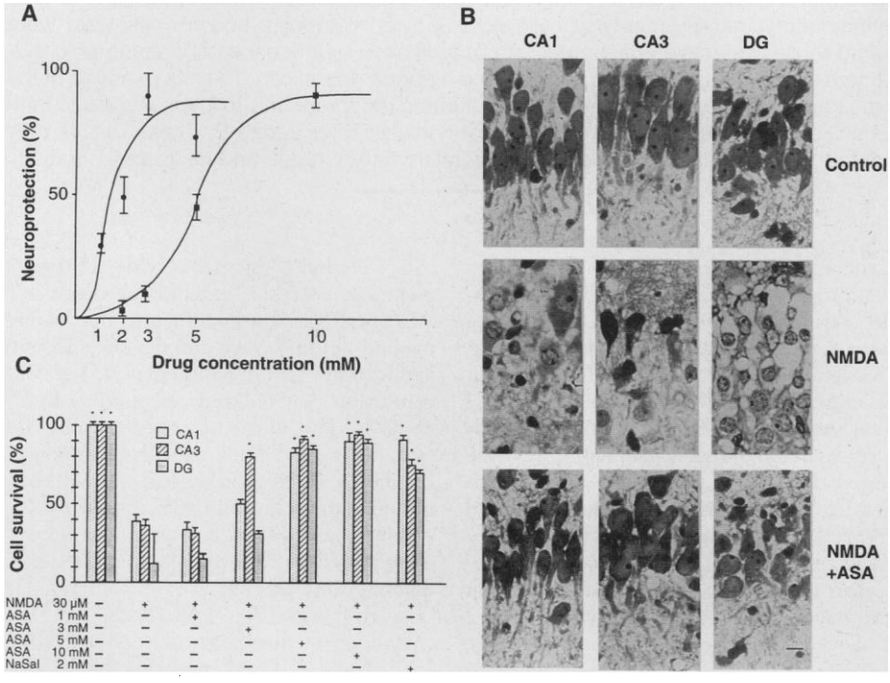

Fig. 1. Neuroprotection by salicylates. (**A**) Concentration-dependent effect elicited by ASA and NaSal in rat cerebellar granule cells. A glutamate (50 μM) pulse was applied in the absence or presence of ASA (●) and NaSal (■). Neuronal survival was expressed as percent of neuroprotection, with glutamate inducing 78 ± 3% of cell loss. The x axis represents drug concentrations. Points represent the means ± SEM of six experiments, run in triplicate, on different culture preparations. (**B**) Prevention of excitotoxic effect of NMDA in rat hippocampal slices by ASA. Sections were exposed to vehicle (control), 30 μM NMDA (NMDA), or 30 μM NMDA and 5 mM ASA (NMDA + ASA). Cell viability was evaluated in CA1, CA3, and DG. Scale bar, 10 μm. (**C**) Effect of ASA and NaSal on NMDA-induced cell loss in rat hippocampal slices. Test drugs were added to the slices at the indicated concentrations and cell viability in CA1, CA3, and DG was analyzed. Columns represent the means ± SEM of three experiments run on four slices each. Differences compared with NMDA alone were significant at $P < 0.01$ as indicated by an asterisk.

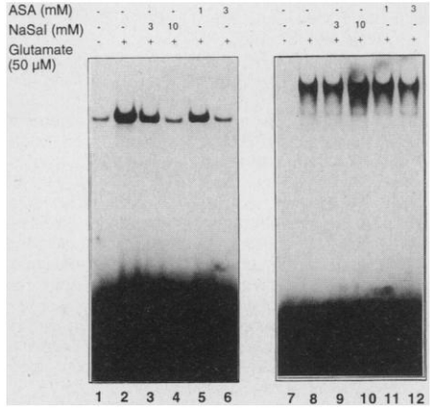

Fig. 3. Effect of neuroprotective concentrations of ASA and NaSal on glutamate-induced NF-κB and AP-1 DNA binding activities. Nuclear extracts from rat cerebellar granule cells were subjected to an electrophoretic mobility-shift assay with γ-^{32}P–labeled oligonucleotide probes containing the immunoglobulin κB (lanes 1 to 6) and the AP-1 DNA binding sites (lanes 7 to 12) (15). Cells were either unstimulated (lanes 1 and 7) or stimulated with 50 μM glutamate (15-min pulse) in the absence (lanes 2 and 8) or presence (lanes 3 to 6 and 9 to 12) of the drugs as indicated.

inhibited the glutamate-induced increase of NF-κB activity in a concentration-dependent manner (Fig. 3), with calculated EC_{50} values of 1.3 and 6 mM for ASA and NaSal, respectively. Parallel experiments in which cell viability was measured 24 hours later revealed a strict correlation between neuroprotective concentrations of anti-inflammatory drugs and blockade of NF-κB induction (EC_{50} values of 1.5 mM for ASA and 5.8 mM for NaSal). The salicylate effect on NF-κB/Rel proteins was specific. In fact, ASA and NaSal failed to modify the glutamate-mediated nuclear induction of the transcriptional complex AP-1 (Fig. 3).

Thus, at concentrations compatible with amounts in plasma during treatment of chronic inflammatory states, salicylates prevented glutamate-induced neurotoxicity. The neuroprotective effect correlated neither with the anti-inflammatory properties of these compounds nor with cyclooxygenase inhibition. In fact, indomethacin exerted anti-inflammatory but not neuroprotective properties, and NaSal was neuroprotective but did not interfere with cyclooxygenase activity (*3*). The common molecular target for ASA and NaSal but not for indomethacin (*10*, *16*) was the blockade of NF-κB induction, suggesting a link between neuroprotection and the nuclear event.

Here we provide evidence for an unusual pharmacological effect of ASA and its metabolite NaSal. In view of their distinct ability to act not merely as anti-inflammatory compounds but also as neuroprotective agents against excitotoxicity, these drugs appear to possess a wider pharmacological spectrum than other nonsteroidal anti-inflammatory drugs.

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16. Indomethacin (1 to 20 μM) was tested for the ability to interfere with glutamate-induced NF-κB activation in cerebellar granule cells. No inhibition was observed. M. Grilli, M. Pizzi, M. Memo, P. F. Spano, unpublished material.

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Tricorn Protease—The Core of a Modular Proteolytic System

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Large macromolecular assemblies have evolved as a means of compartmentalizing reactions in organisms lacking membrane-bounded compartments. A tricorn-shaped protease was isolated from the archaeon *Thermoplasma* and was shown to form a multisubunit proteolytic complex. The 120-kilodalton monomer assembled to form a hexameric toroid that could assemble further into a capsid structure. Tricorn protease appeared to act as the core of a proteolytic system; when it interacted with several smaller proteins, it displayed multicatalytic activities.

In vivo proteolysis is an essential element of many regulatory processes. It must be subject to spatial and temporal control in order to prevent damage to the cell. Prokaryotic cells, which lack membrane-bounded compartments, have developed large macromolecular assemblies or "molecular organelles" so as to confine proteolysis to an inner cavity to which only proteins targeted for degradation have access. The paradigm of such a proteolytic complex is the proteasome (*1*), which is ubiquitous across the three urkingdoms archaea (*2*), bacteria (*3*, *4*) and eukarya (*5*). In the course of searching for regulatory components of the proteasome (*6*) in *Thermoplasma acidophilum*, we discovered a proteolytic complex of high molecular mass that is not related to the proteasome. This complex seems to be the core of a modular proteolytic system generating multicatalytic activities.

We purified the high-molecular-weight (HMW) protein to homogeneity by a sequence of chromatography steps (*7*). The purified protein migrates at 720 kD in gel filtration chromatography (versus migration at 680 kD by the 20S proteasome), and it turned out to be composed of a single polypeptide of 120 kD when subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The purified protein

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