Journal of Neurochemistry
Raven Press, Ltd., New York

Blood–Brain Barrier Breakdown in Cold-Injured Brain Is Linked to a Biphasic Stimulation of Ornithine Decarboxylase Activity and Polyamine Synthesis: Both Are Coordinately Inhibited by Verapamil, Dexamethasone, and Aspirin

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Abstract: An early increase in ornithine decarboxylase (ODC) activity and polyamine levels in rat cerebral capillaries was previously implicated in the mediation of blood–brain barrier (BBB) breakdown in cold-injured brain. A time course study in rat cerebrum indicated that cold injury evokes a biphasic increase in ODC activity and polyamine levels in perilesional cortex. ODC activity rose sharply (fourfold) within 1 min, remained elevated for 5 min, and then returned to the basal level by 10 min. A transient rise in polyamine concentration followed in the rank order of putrescine > spermidine > spermine. A secondary rise in ODC activity commenced in perilesional tissue at 2–6 h and peaked (8.8-fold) at 48 h. Major increases in the content of putrescine (330%), spermidine (103%), and spermine (50%) developed at 48–72 h. α-Difluoromethylornithine (DFMO), a specific irreversible inhibitor of ODC, suppressed the evoked increase in ODC activity and abolished the associated increase in content of polyamines, findings indicating that the accumulation of polyamines in cryoinjured brain reflects enhanced synthesis resulting from an ODC-mediated increase in putrescine content. Cycloheximide and actinomycin D were without effect on the early increase in ODC activity but inhibited the delayed increase in ODC activity, an observation suggesting that the initial increase in activity reflects an activation of a cryptic ODC via a posttranslational process, whereas the delayed increase in activity results from ODC synthesis mainly under transcriptional control. Because membrane phospholipid degradation, release of diacylglycerol and free arachidonic acid, and prostaglandin formation are early events in cold-injured brain, we assessed the effects of verapamil (a calcium channel blocker), dexamethasone (which inhibits arachidonic acid release), and aspirin (a cyclooxygenase inhibitor). These agents resembled DFMO in that they inhibited the early (2-min) and delayed (24-h) increase in ODC activity and polyamine concentrations and concurrently attenuated BBB breakdown in the perilesional cortex, as monitored by fluorescein transport. Exogenous putrescine nullified the protective effect of verapamil, dexamethasone, and aspirin on BBB breakdown following cryogenic injury. These results implicate Ca^{2+} influx via calcium channels, phospholipid hydrolysis, and prostaglandin synthesis in cryogenically induced stimulation of ODC activity and further strengthen the evidence linking polyamines to BBB breakdown. Changes in ODC-regulated polyamine synthesis in brain cells may play an important role in other aspects of the pathophysiology of cerebral injury. **Key Words:** Ornithine decarboxylase—Polyamines—Putrescine—Brain injury—Brain edema—Calcium—Prostaglandins—Blood–brain barrier breakdown—Verapamil—Aspirin — α - Difluoromethylornithine — Dexamethasone. **Koenig H. et al.** Blood–brain barrier breakdown in cold-injured brain is linked to a biphasic stimulation of ornithine decarboxylase activity and polyamine synthesis: Both are coordinately inhibited by verapamil, dexamethasone, and aspirin. *J. Neurochem.* **52,** 101–109 (1989).

Cold injury of the cerebral cortex has been extensively used for induction of focal blood–brain barrier (BBB) breakdown and spreading vasogenic brain edema (Chan et al., 1983; Vorbrodt et al., 1985). In this experimental model, abnormal microvascular permeability develops acutely (<1–5 min) in the margin of the cortical lesion (Yamamoto et al., 1976; Chan et al., 1983; Trout et al., 1986) and results in a pro-

Received November 23, 1987; final revised manuscript received July 6, 1988; accepted July 7, 1988.

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Portions of this work have been presented elsewhere in abstract form (Koenig et al., 1984; Lu et al., 1984; Goldstone et al., 1985).

Abbreviations used: BBB, blood–brain barrier; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase.

gressive white matter edema in the lesioned hemisphere, which peaks at 24–48 h (Klatzo et al., 1980). Cold injury also induces changes in neuronal function and metabolism that appear to be unrelated to cerebral edema. These include epileptic discharges and slow (delta) wave activity in the electroencephalogram (Pappius and McCann, 1969; Lewin, 1972) and a depression in local cerebral glucose utilization appearing hours to days following injury (Pappius, 1981).

We recently found that focal cold injury of rat cerebral cortex induces an early increase in the levels of the polyamines putrescine, spermidine, and spermine and of the activity of their rate-regulating synthetic enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) in cerebral capillaries and brain cells in the lesioned hemisphere (Koenig et al., 1983*a*). α-Difluoromethylornithine (DFMO), a specific, irreversible, enzyme-activated inhibitor of ODC (Metcalf et al., 1978; Seely et al., 1982), blocks the accumulation of polyamines and the abnormal microvascular permeability, and putrescine, the product of ODC activity, reverses the effects of DFMO, thus indicating that newly synthesized polyamines play an essential role in BBB breakdown (Koenig et al., 1983*a*). A subsequent study showed that the increase in ODC activity and polyamine concentrations correlates closely with the leakage of tracers, e.g., fluorescein and horseradish peroxidase, in topographic distribution and rapidity of onset and is associated with an acute, DFMO-sensitive stimulation of endocytosis, vesicular transport, and microvillus formation in capillary endothelium (Trout et al., 1986).

We investigated the time course of the ODC activity and polyamine content changes in this experimental model. The results presented here reveal that cold injury induces a biphasic stimulation of ODC activity and polyamine synthesis in perilesional tissue. In the first phase, there is a rapid, transient increase in ODC activity and polyamine levels between 1 and 5 min. A secondary rise in ODC activity and polyamine levels occurs after a lag period of ~4 h and peaks at 48 h. Other early events in cold-injured brain tissue include membrane phospholipid degradation (Chan et al., 1983) and large increases in content of free arachidonic acid and other fatty acids (Chan et al., 1983; Pappius and Wolfe, 1983; Wolfe and Pappius, 1983) and diacylglycerol (Politi et al., 1985). We have examined the hypothesis that these membrane phospholipid changes, which are initiated in part by Ca^{2+}-dependent phospholipases (Bazan and Rodriguez de Turco, 1980; Wolfe, 1982; Chan et al., 1983; Bazan et al., 1984; Politi et al., 1985), may be implicated in the increase in brain ODC activity and BBB breakdown induced by cold injury. To this end, we used verapamil, a calcium channel blocker (Kohlhardt et al., 1972); dexamethasone, a synthetic glucocorticoid inhibiting phospholipase-mediated arachidonate release (Hong and Levine, 1976; Wolfe, 1982); and aspirin, a cyclooxygenase inhibitor (Vane, 1971). The results show that these agents are effective in suppressing the early and delayed increases in ODC activity and the enhanced microvascular permeability induced by cold injury. The protective effect of these agents was reversed by exogenous putrescine.

MATERIALS AND METHODS

Cryogenic injury

Albino Sprague–Dawley rats weighing 210–260 g were anesthetized with trichloroethylene vapor during the operative procedures. Two holes 3 mm in diameter were drilled in the right parietal region of the skull, and the exposed dura was covered with Saran Wrap. A standard cryogenic injury yielding a reproducible superficial necrotic zone was produced in the parietal cortex by a 30-s application to the dura of two metal probes precooled in liquid nitrogen as described elsewhere (Koenig et al., 1983*a*; Trout et al., 1986). Rats were killed, and brain metabolism was rapidly quenched at each time point (measured after beginning the cryogenic injury) by immersing the head in liquid nitrogen for 30 s. The head was immediately severed, the brain was removed and placed on ice, and the perilesional tissue (amounting to ~10% of the right hemisphere wet weight, corresponding to the zone of in vivo staining by sodium fluorescein), was excised (see Fig. 1 in Trout et al., 1986) and stored at −70°C until used.

Analytical methods

For polyamine content measurements, tissue samples were homogenized in 5–10 volumes of 0.2 *M* perchloric acid and centrifuged at 10,000 *g* for 10 min. The polyamines in the supernatant were dansylated, separated by TLC, and quantified by spectrofluorometry (Seiler, 1983). The dansylated derivatives of putrescine, spermidine, and spermine were separated by one-dimensional TLC using the following procedure. The samples were applied to Analtech Unisil G precoated TLC plates (250 μm thick) and chromatographed successively in benzene/cyclohexane/methanol (85:15:15 by volume), cyclohexane/ethyl acetate (3:2 vol/vol), and finally twice in cyclohexane/ethyl acetate (1:2 vol/vol). The plates were all chromatographed in one direction and were allowed to dry between individual runs. The assay was linear over a wide range of polyamine concentrations (0.05–100 nmol/30 μl), with a coefficient of variation of 3% (at 0.1 and 1 nmol/30 μl) and a limit of detection of 25 pmol.

ODC activity was measured by a modification (Koenig et al., 1983*b*) of the method of Djurhuus (1981) and expressed as pmol of putrescine/h. Tissue samples were homogenized in ~5 volumes of cold homogenizing medium, containing 50 m*M* sodium phosphate buffer (pH 7.2), 5 m*M* dithiothreitol, 0.1 m*M* EDTA, and 50 μ*M* pyridoxal phosphate. Homogenates were centrifuged at 12,000 *g* for 20 min at 4°C, and the pellets were discarded. To 0.1 ml of supernatant was added 0.1 ml of incubation medium containing 5 m*M* dithiothreitol, 0.2 m*M* pyridoxal phosphate, 50 m*M* sodium phosphate buffer (pH 7.2), 0.1 m*M* L-ornithine, and 0.3 μCi L-[3-^{3}H(*n*)]ornithine. The tubes were incubated for 2 h at 37°C and then chilled to 0°C. Forty microliters of each sample was applied to Whatman P-81 cation-exchange paper, and the paper strips were subjected to descending chromatography for 2 h using 0.1 *M* NH_4OH as the eluant. The paper strips were dried, and the spots corresponding to putrescine standard, which was detected by ninhydrin, were cut out, placed in scintillation vials containing 1 ml of deionized

water, and heated at 40°C for 2 h. In this system, putrescine remains at the origin, and ornithine is eluted off the paper. Ten milliliters of Aquassure scintillation fluid was added to the vials, and the radioactivity was counted in a Beckman LS-250 liquid scintillation counter. Enzyme controls containing the complete assay mixture and boiled enzyme were processed with each determination, and these values were subtracted from those of the test samples. Protein content was determined according to the technique of Lowry et al. (1951). In the range in which measurements were made, ODC activity was linear with time and protein content.

Fluorescein uptake

Cerebral microvascular permeability was measured fluorimetrically with intravenously administered fluorescein. Fluorescein occurs mostly free in the blood and behaves as a micromolecular tracer in defining sites of BBB breakdown (Wolman et al., 1981). Sodium fluorescein (10% in physiological saline, 1 ml/kg of body weight) was injected into the caudal vein with the rat under Nembutal anesthesia 5 min before the animal was killed. Approximately 2 ml of blood was withdrawn from the heart, and the brain was perfused with saline injected through the left ventricle to flush out intravascular fluorescein. Fluorescein in 7.5% trichloroacetic acid extracts of brain samples was quantified by fluorimetry at an excitation wavelength of 440 nm and an emission wavelength of 525 nm as previously described (Trout et al., 1986). Serum fluorescein concentrations were similarly determined. Fluorescein uptake was expressed as (μg of fluorescein/mg of protein)/(μg of fluorescein/μl of blood) to normalize uptake values for blood levels of the dye at time of killing.

Experimental protocol

The first two experiments investigated the time course of the changes in ODC activity and polyamine levels in the perilesional cortex at several times between 1 min and 72 h after cryogenic injury as described above.

In subsequent experiments, three treatment protocols were used. In the first protocol, rats received cycloheximide (20 mg/kg), actinomycin D (2 mg/kg), or saline intraperitoneally 5 min before cold injury and were killed 2 min later or received cycloheximide, actinomycin D, or saline 30 min before and 2 h after cold injury and were killed 6 h after injury. In the second protocol, the following treatment groups were used: (a) unoperated, vehicle-injected controls; (b) sham-operated (anesthetized, skull-drilled, warm probe-applied), vehicle-injected controls; (c) vehicle-injected, cold-injured controls; and (d) cold-injured animals treated with dexamethasone (2 mg/kg of body weight), aspirin (50 mg/kg), verapamil (25 mg/kg), or DFMO (500 mg/kg). For the 2-min experiment, animals received drugs or vehicle by intraperitoneal injection 10 min before cryogenic injury. For the 24-h experiment, animals received a second dose of drug or vehicle at 7 h and were killed 24 h after injury. In the third treatment protocol, cold-injured rats received verapamil, dexamethasone, and aspirin without or with putrescine dihydrochloride (100 mg/kg) by intraperitoneal injection 10 min before cryogenic injury and were killed 2 min after injury.

Data were expressed as mean ± SEM values. Results were analyzed statistically by a one-way analysis of variance using the program designed for for the R&M Biometrics (Nashville, TN, U.S.A.) Bioquant II System. Fisher's least significant difference test was used to assess the statistical significance of differences between individual group means (Kirk, 1982).

RESULTS

ODC activity and polyamine levels

As has been repeatedly noted (Seiler, 1982), the basal activity of ODC in adult rat cerebrum is relatively low (6–16 pmol/h/mg of protein). The time course of ODC changes in the perilesional tissue is shown in Fig. 1. ODC activity in the perilesional cortex was increased more than fourfold 1 min after initiating cold injury, remained elevated for 5 min, and returned to near basal values by 10 min. Small increases (50–90%) in ODC activity were seen between 30 min and 4 h, followed by a larger increase (fourfold) at 6 and 24 h, which peaked (8.8-fold) by 48 h but was still elevated (4.4-fold) at 72 h. Sham injury (animals anesthetized, drill holes made, and warm probes applied) induced a 34% increase ($p < 0.05$) in ODC activity at 2 min and a 47% increase ($p > 0.05$) at 24 h (Table 1). These changes were only 22–24% of the increment in ODC activity evoked by application of the cold probe at these times, a finding indicating that cryogenic injury accounts for some 80% of the increase in ODC activity. Dienel and Cruz (1984) previously reported that drilling burr holes in rat skull increased brain ODC activity after 9 h.

The time course of polyamine content changes induced by cold injury is shown in Fig. 2. The concentration of all three polyamines in perilesional tissue increased abruptly 1 min after cold injury, peaked between 2 and 5 min (2.6-, 1.8-, and 1.7-fold for putrescine, spermidine, and spermine, respectively), and subsequently declined to basal values by 10–60 min. A secondary rise in polyamine levels commenced at ~2–4 h but did not attain significance until 24 h for putrescine and 48 h for spermidine and spermine. The content of all three polyamines increased maximally at 48–72 h. Sham injury was associated with small increases in levels of polyamines that were statistically insignificant (Table 1).

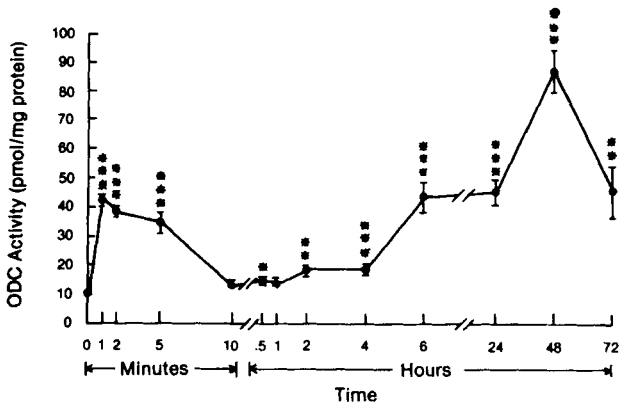

FIG. 1. Time course of ODC activity changes in the perilesional tissue after cold injury. Data are mean ± SEM (bars) values (n = 3). ODC activity is expressed as pmol/h/mg of protein. The significance of differences from the control is indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE 1. *Effect of verapamil, dexamethasone, aspirin, and DFMO on ODC activity and polyamine levels in perilesional tissue after cryogenic injury*

Treatment	ODC activity (pmol/h/mg of protein)	nmol/mg of protein		
		Putrescine	Spermidine	Spermine
Experiment A (2 min after injury)				
Unoperated control	15.9 ± 0.45[a]	0.08 ± 0.003	0.43 ± 0.02	0.78 ± 0.05
Sham-operated control	21.3 ± 1.6	0.10 ± 0.01	0.42 ± 0.07	0.88 ± 0.03
Injury control	40.4 ± 3.07[b]	0.38 ± 0.02[b]	0.70 ± 0.01[b]	1.24 ± 0.06[b]
Verapamil	24.2 ± 2.9[c]	0.13 ± 0.02[d]	0.55 ± 0.05[e]	1.05 ± 0.02[e]
Dexamethasone	28.4 ± 2.1[c]	0.14 ± 0.003[d]	0.55 ± 0.01[e]	0.97 ± 0.04[e]
Aspirin	24.1 ± 0.5[c]	0.12 ± 0.01[d]	0.52 ± 0.02[c]	0.91 ± 0.10[c]
DFMO	25.6 ± 1.2[c]	0.11 ± 0.01[d]	0.40 ± 0.01[d]	0.72 ± 0.03[d]
Experiment B (24 h after injury)				
Unoperated control	16.7 ± 1.2	0.12 ± 0.01	0.74 ± 0.005	1.34 ± 0.02
Sham-operated control	24.5 ± 3.3	0.11 ± 0.06	0.66 ± 0.08	1.21 ± 0.13
Injury control	49.5 ± 5.9[a]	0.47 ± 0.009[b]	0.96 ± 0.01[a]	1.75 ± 0.04[a]
Verapamil	24.5 ± 1.8[d]	0.32 ± 0.02[d]	0.74 ± 0.05[c]	1.36 ± 0.13[e]
Dexamethasone	29.8 ± 2.5[d]	0.35 ± 0.01[d]	0.88 ± 0.05	1.51 ± 0.08
Aspirin	28 ± 1.4[d]	0.32 ± 0.02[d]	0.73 ± 0.07[c]	1.45 ± 0.03[e]
DFMO	20.7 ± 0.9[d]	0.10 ± 0.006[d]	0.35 ± 0.01[d]	0.63 ± 0.01[d]

Female rats were untreated (unoperated control), sham-operated, or subjected to cryogenic injury in the right parietal area. Experiment A, treated rats received verapamil (25 mg/kg), dexamethasone (2 mg/kg), aspirin (50 mg/kg), DFMO (300 mg/kg), or vehicle (injury control) intraperitoneally 10 min before cryogenic injury, and animals were killed 2 min after termination of cryogenic injury. In Experiment B, animals received a second dose of the inhibitors 7 h after injury and were killed 24 h after the injury. Data are mean ± SEM values (n = 3–4).

Significance of difference versus sham-operated control: [a]$p < 0.05$, [b]$p < 0.001$.

Significance of difference versus injury control: [c]$p < 0.01$, [d]$p < 0.001$, [e]$p < 0.05$.

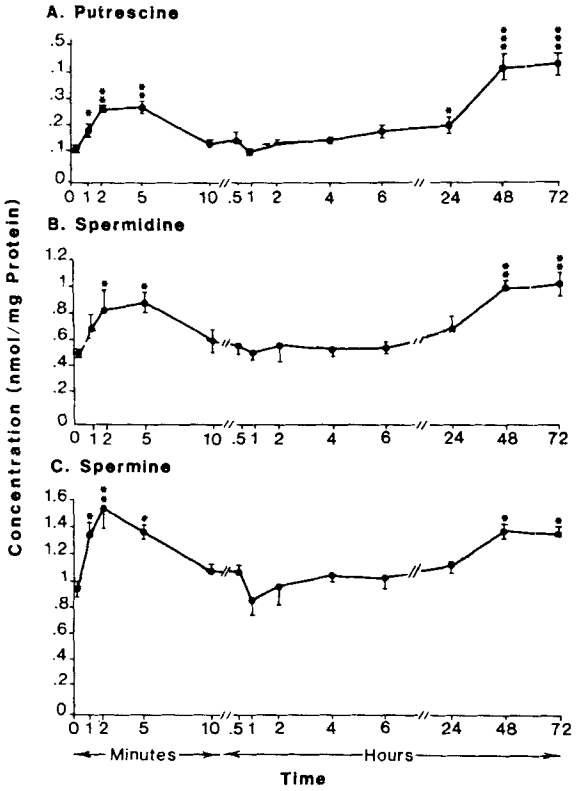

FIG. 2. Time course of polyamine content changes in the perilesional tissue after cold injury. Data are mean ± SEM (bars) values (n = 3). The significance of differences from the control is indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Effect of cycloheximide and actinomycin D

Table 2 shows that the early increase in cortical ODC activity evoked by cryogenic injury is insensitive to cycloheximide and actinomycin D. However, cycloheximide abolished the increase in ODC activity observed 6 h after injury, and actinomycin D caused a 60% inhibition in this increase. Neither agonist altered the brain protein concentration in these experiments. These data suggest that the early stimulation of ODC activity is independent of protein and RNA synthesis, whereas the delayed increase in ODC activity involves de novo synthesis of ODC protein that is mediated, at least in part, by new mRNA synthesis.

Effect of DFMO, verapamil, dexamethasone, and aspirin

To assess the potential mechanisms underlying the early and delayed increases in ODC activity and polyamine content and the concomitant changes in microvascular permeability, brains were examined at 2 min and 24 h after cryogenic injury. These experiments confirmed that cryogenic injury induced a major increase in ODC activity and levels of putrescine, spermidine, and spermine in perilesional tissue at 2 min and 24 h (Table 1). Dexamethasone, aspirin, and verapamil inhibited the cold injury-induced stimulation of ODC activity by 62–85% and effectively blocked the accumulation of all three polyamines at 2 min (Table 1). Dexamethasone, aspirin, and verapamil also inhibited the delayed increase in ODC activity and putres-

TABLE 2. *Effect of cycloheximide and actinomycin D on stimulation of ODC activity evoked in rat cerebrum by cold injury*

Treatment	ODC activity (pmol/h/mg of protein)
Unoperated control	6.4 ± 0.3
Injury control	
2 min	12.2 ± 0.2[a]
6 h	21.9 ± 1.1[a]
Cycloheximide	
2 min	11.8 ± 0.3[a]
6 h	5.0 ± 0.4[b]
Actinomycin D	
2 min	12.8 ± 0.8[a]
6 h	12.6 ± 2.2[a,b]

Rats received cycloheximide (20 mg/kg), actinomycin D (2 mg/kg), or saline intraperitoneally 5 min before cold injury and were killed 2 min later or received cycloheximide, actinomycin D, or saline 30 min before and 2 h after cold injury and were killed 6 h after injury. Lesioned cerebral hemispheres were rapidly excised and assayed for ODC activity as described in Materials and Methods. Data from the unoperated controls receiving vehicle in the 2-min and 6-h groups were similar and are, therefore, reported as a single value (n = 6). Data are mean ± SEM values (n = 6 for unoperated control and n = 3 for experimental animals).

[a] $p < 0.001$ versus unoperated control.

[b] $p < 0.001$ versus injury control.

cine concentration 24 h after cold injury (Table 1). Aspirin and verapamil attenuated the increase in spermidine and spermine content, but dexamethasone was less effective in this respect (Table 1). DFMO caused a 72% inhibition of evoked ODC activity (Table 1) and blocked the increase in polyamine content at 2 min (see Fig. 2 in Trout et al., 1986). At 24 h, DFMO decreased the ODC activity and polyamine levels to below sham control values (Table 1).

Fluorescein transport

Microvascular permeability was assessed by measuring fluorescein transport into perilesional cortex 5 min after administration of an intravenous bolus of the dye. Fluorescein uptake was minimal in unoperated controls but increased 28-fold in perilesional tissues at 2 min and 15-fold at 24 h after cold injury (Table 3). Sham injury caused a sixfold increase in fluorescein uptake in brain cortex subjacent to the cranial perforations at 2 min, presumably as a consequence of local heat generated by the drilling. By 24 h, however, fluorescein transport was no longer increased in sham-injured controls (Table 3).

Dexamethasone, aspirin, verapamil, and DFMO effectively suppressed the cold injury-evoked increase in fluorescein permeability measured at 2 min and 24 h following cold injury (Table 3). Thus, the ability of these agents to inhibit the stimulation of ODC activity and polyamine accumulation induced by cryogenic injury correlated closely with their ability to attenuate fluorescein transport in the perilesional cortex. To examine further the putative role of polyamine depletion in protection against BBB breakdown, we investigated the effect of exogenous putrescine (100 mg/kg) when coadministered with verapamil, dexamethasone, and aspirin (Table 4). Putrescine reversed the protective effect of these agents and restored the increase in fluorescein transport into perilesional tissue evoked by cold injury at 2 min. We previously showed that exogenous putrescine replenishes cerebral microvascular polyamines and reverses the protective effect of DFMO in cryogenically induced BBB breakdown (Koenig et al., 1983*a*).

TABLE 3. *Effect of verapamil, dexamethasone, aspirin, and DFMO on fluorescein transport in perilesional tissue after cryogenic injury*

Treatment	Fluorescein transport [(μg of fluorescein/mg of protein)/(μg of fluorescein/μl of serum)] 2 min after injury	24 h after injury
Sham-operated control	0.17 ± 0.02	0.040 ± 0.003
Unoperated control	0.029 ± 0.007[a]	0.026 ± 0.004
Injury control	0.82 ± 0.24[a]	0.38 ± 0.01[b]
Verapamil	0.064 ± 0.008[c]	0.14 ± 0.02[d]
Dexamethasone	0.18 ± 0.06[d]	0.13 ± 0.04[d]
Aspirin	0.11 ± 0.01[c]	0.13 ± 0.01[d]
DFMO	0.11 ± 0.06[c]	0.09 ± 0.03[c]

The experimental protocol is given in Table 1. Fluorescein was injected into the tail vein 5 min before the animals were killed, and cryogenic injury was initiated 3.5 min or 24 h before death. Data are mean ± SEM values (n = 3).

Significance of difference versus sham-operated control: [a] $p < 0.01$, [b] $p < 0.001$.

Significance of difference versus injury control: [c] $p < 0.001$, [d] $p < 0.01$.

TABLE 4. *Putrescine reverses inhibition by verapamil, dexamethasone, and aspirin of fluorescein transport into perilesional tissue after cryogenic injury*

Treatment	Fluorescein transport [(μg of fluorescein/mg of protein)/(μg of fluorescein/μl of serum)]
Verapamil	0.113 ± 0.009
Verapamil + putrescine	0.870 ± 0.247[a]
Dexamethasone	0.145 ± 0.035
Dexamethasone + putrescine	0.72 ± 0.11[a]
Aspirin	0.090 ± 0.019
Aspirin + putrescine	0.31 ± 0.05[b]

Female rats received verapamil (25 mg/kg), dexamethasone (2 mg/kg), or aspirin (50 mg/kg) without or with putrescine (100 mg/kg) 10 min before cryogenic injury, and animals were killed 5 min after intravenous fluorescein administration and 2 min after termination of cryogenic injury. Data are mean ± SEM values (n = 3).

Significance of difference versus agent without putrescine: [a] $p < 0.05$, [b] $p < 0.01$.

DISCUSSION

These experiments have revealed a previously unrecognized biphasic stimulation of ODC activity in perilesional brain tissue following a cryogenic injury. In the primary phase, ODC activity increases abruptly to a maximal value (fourfold) at 1 min (the earliest time point examined), remained high for 5 min, and decreased to the resting level by 10 min. This transient increase in ODC activity was promptly followed by a transient elevation in the concentration of putrescine, spermidine, and spermine. Thus, levels of all three polyamines were increased at 1 min. Cell injury was the effective stimulus in this model, as drilling holes in the skull of anesthetized rats without applying the cold probe induced a much smaller increase in ODC activity and negligible increases in polyamine levels. That the stimulation of ODC activity was instrumental in initiating the surge of polyamine content is suggested by the finding that the level of putrescine, the first molecule of the polyamine synthetic pathway and the direct product of ODC, increased most rapidly and to the greatest extent (180, 260, and 270% of the control at 1, 2, and 5 min, respectively, compared with corresponding values of 141, 169, and 180% for spermidine and 146, 167, and 180% for spermine). Administration of DFMO, a suicide inhibitor of ODC (Metcalf et al., 1978; Seely et al., 1982), prevented the rapid increase in level of polyamines, thereby confirming that the accumulation of polyamines in perilesional tissue is a direct consequence of the cryoinjury-induced stimulation of ODC activity, i.e., putrescine production is rate limiting for the biosynthesis of the higher polyamines. However, these data do not demonstrate the precursor–product relationship of spermidine and spermine, apparently because of the very rapid interconversion of these polyamines. Paradoxically, the measured ODC activity, 0.7 pmol/min/mg of protein, is much too low to account for the measured increment of polyamines, 1,000 pmol/mg of protein during the first 5 min following cold injury. This discrepancy between ODC activity and ODC product has been previously noted in cold-injured cerebral cortex (Trout et al., 1986) and is evident in rapid responses to various other stimuli (Koenig et al., 1983*b,c*, 1987; Iqbal and Koenig, 1985). These observations suggest that the ODC activity measured in vitro in broken cell preparations greatly underestimates the catalytic activity of ODC in stimulated, intact cells. The basis of this disparity remains to be clarified.

The transience of the initial increase in ODC activity is considered to reflect a cryoinjury-induced activation–deactivation cycle involving a cryptic ODC in brain microvascular and neural cells (Koenig et al., 1983*a*; Trout et al., 1986). It is significant in this context that only a small portion of the ODC, ~2% of the total, in brain cytosolic extracts is catalytically active in a conventional ODC assay, owing, in part at least, to complexation with ODC antizyme (Laitinen et al., 1986). The early stimulation of ODC activity was insensitive to cycloheximide and actinomycin D, an observation indicating that the increased ODC activity is not dependent on protein or RNA synthesis.

The secondary rise in ODC activity in the perilesional cortex was well developed between 2 and 6 h and peaked (8.8-fold) at 48 h. Although the content of putrescine and the other polyamines underwent only modest, statistically insignificant increases during the first 6 h, a major increase in level of putrescine (330%), spermidine (105%), and spermine (50%) developed by 48–72 h. DFMO suppressed the increase in ODC activity and the concomitant accumulation of polyamines at 24 h. Thus, the time course, rank order, and DFMO sensitivity of the late accumulation of polyamines in cryoinjured cerebrum are all consistent with enhanced polyamine synthesis via the ODC-regulated pathway. The secondary increase in ODC activity appears to be a consequence of enhanced de novo synthesis of ODC, which is regulated, in part at least, by transcription of new mRNA, as it was prevented by cycloheximide and substantially inhibited by actinomycin D. Dienel and Cruz (1984) found a small increase in ODC activity in cold-injured cerebrum at 9 h.

These experiments have shown that verapamil, dexamethasone, and aspirin resemble DFMO in their ability to suppress the early (2-min) and delayed (24-h) stimulation of ODC activity, accumulation of polyamines, and BBB breakdown induced in rat cerebrum by cold injury. Because these agents should have no direct effect on ODC, it may be assumed that they act indirectly by inhibiting one or more prior biochemical events essential for the stimulation of ODC activity. It is known that the breakdown of phospholipids (Chan et al., 1983; Bazan et al., 1984) and the resultant accumulation of free arachidonic acid (Chan et al., 1983; Pappius and Wolfe, 1983; Wolfe and Pappius, 1983; Bazan et al., 1984), prostaglandins (Pappius and Wolfe, 1983; Wolfe and Pappius, 1983), and diacylglycerol (Bazan et al., 1984; Politi et al., 1985) are among the earliest biochemical changes of membrane components occurring in cold-injured cortex. In addition, free oxygen radicals have been implicated in BBB breakdown (Chan et al., 1984) associated with enhanced arachidonate metabolism via cyclooxygenase (Wei et al., 1986), which generates superoxide in the presence of suitable reducing cosubstrates such as NADH and NADPH (Kukreja et al., 1986). The agents used in the present study would be expected to interfere with one or more of these changes. In nonneural tissues, glucocorticoids exert antiinflammatory actions by decreasing arachidonate release (Hong and Levine, 1976) through induction of a phospholipase A_2-inhibitory protein or proteins, variously designated as lipocortin (Flower and Blackwell, 1979; Flower, 1981) and lipomodulin (Hirata et al., 1980). Aspirin and related nonsteroidal antiinflammatory agents inhibit the for-

mation of prostaglandins from arachidonate by blocking the action of cyclooxygenase (Vane, 1971) and would also decrease free radical production by this pathway. Verapamil is a calcium antagonist that specifically blocks the entry of Ca^{2+} into excitable cells through slow calcium channels (Kohlhardt et al., 1972).

In cryoinjured brain, a low dose (0.25 mg/kg) of dexamethasone did not inhibit the acute release of arachidonic acid in the lesion, although it ameliorated the depression of cerebral glucose utilization (Pappius, 1982; Pappius and Wolfe, 1983) and the EEG abnormality that develop subsequently (Sutton et al., 1980). However, a larger dose (1.25 mg/kg) of dexamethasone abolished the release of arachidonic acid and inhibited the accumulation of diacylglycerol in cryoinjured brain (Politi et al., 1985). Indomethacin, another cyclooxygenase inhibitor, blocked acute prostaglandin synthesis in the lesion and was more effective than dexamethasone in enhancing cerebral glucose metabolism in cold-injured brain (Pappius and Wolfe, 1983). The effects of verapamil or other calcium channel blockers do not appear to have been previously studied in cryogenic brain injury but could conceivably attenuate Ca^{2+}-dependent reactions, including phospholipase A_2 activation, involved in phospholipid degradation and metabolism.

The rapidity of the initial transient stimulation of ODC activity in cryoinjured cerebral cortex and its cycloheximide insensitivity point to an activation/deactivation of a latent ODC by a posttranslational process (Koenig et al., 1983*a*, 1988; Trout et al., 1986). The present report shows that this rapid ODC activation/deactivation cycle is inhibited by verapamil, dexamethasone, and aspirin, as well as by the suicide ODC inhibitor DFMO. These findings suggest that enhanced Ca^{2+} influx through calcium channels, phospholipid hydrolysis, arachidonic acid release, prostaglandin synthesis, and possibly also free oxygen radicals are important early events in the membrane cascade leading to ODC activation/deactivation. However, the precise cellular and molecular mechanisms underlying the rapid regulation of ODC activity remain to be clarified. The delayed increase in ODC activity was also inhibited by verapamil, dexamethasone, aspirin, and DFMO. The long-term increase in ODC activity appears to be a consequence of transcriptional regulation of ODC synthesis, as it was inhibited by cycloheximide and actinomycin D. It is not clear from our experiments, however, whether enhanced Ca^{2+} influx, arachidonate release, and prostaglandin synthesis play a direct role in triggering the transcriptional stimulation of ODC synthesis, or whether these agents prevent genomic regulation of ODC by ablating the initial transient activation of ODC.

The finding that verapamil, aspirin, and dexamethasone, as well as DFMO, suppress the rapid and delayed increases in content of polyamines is consistent with the inhibitory effects of these agents on cryogenically induced stimulation of ODC activity. In this context, it is pertinent to note that abnormalities in neuronal function in cold-injured brain, which are manifested by epileptic discharges and delta wave activity in the EEG (Pappius and McCann, 1969), are attenuated by dexamethasone (Sutton et al., 1980). Furthermore, the depression in local cerebral glucose utilization, which is well developed 2–3 days after cold injury (Pappius, 1981), is ameliorated by dexamethasone (Pappius, 1982; Pappius and Wolfe, 1983) and indomethacin (Pappius and Wolfe, 1983). These data, taken together with the present observations, support the inference that polyamines may also be involved in the pathophysiology of neuronal dysfunction in cold-injured brain.

Finally, these experiments have demonstrated that the early and delayed breakdown of the BBB in cryogenically traumatized cerebrum, as measured by fluorescein transport, is prevented by verapamil, dexamethasone, and aspirin, as well as by DFMO. Endogenous glucocorticoids have been shown to regulate the permeability of the BBB to macromolecules (Long and Holaday, 1985). Dexamethasone decreases the permeability of the BBB in normal animals (Hedley-White and Hsu, 1986), as well as in animals with experimentally induced BBB breakdown (Eisenberg et al., 1970; Johansson, 1978; Neuwelt et al., 1982). Our results support the conclusion that glucocorticoids, aspirin, and verapamil protect against BBB breakdown in cryogenically injured brain by interfering with the membrane cascade leading to stimulation of ODC activity and polyamine synthesis in cerebral microvessel endothelium (Koenig et al., 1983*a*; Trout et al., 1986). This conclusion receives additional support from the finding that exogenous putrescine reverses the protective effect of verapamil, dexamethasone, and aspirin, presumably by circumventing ODC inhibition and replenishing depleted microvascular polyamines. Previous studies have shown that putrescine increased levels of cerebral parenchymal and microvascular polyamines and concurrently nullified DFMO protection of cryogenically induced disruption of the BBB (Koenig et al., 1983*a*; Trout et al., 1986). These reversal experiments with exogenous putrescine confirm that verapamil, dexamethasone, and aspirin attenuation of BBB breakdown is a direct consequence of inhibition of polyamine synthesis.

Acknowledgment: We thank Mrs. T. Howell for typing the manuscript. This research was supported by funds from the Veterans Administration Research Service and by grants NS 18047 and HL 26835 from the National Institutes of Health.

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