Calcium Antagonist PY 108–068: Demonstration of Its Efficacy in Various Types of Experimental Brain Ischemia

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SUMMARY The protective properties of PY 108–068 against ischemic disturbances in the brain were investigated in vitro and in vivo. The calcium antagonistic effects were demonstrated on isolated, calcium-loaded arterial rings of feline cerebral arteries. Determination of brain tissue oxygenation with PO2 microelectrodes showed that PY 108–068 improved oxygen supply in the cortex of cats subjected to epicerebral arterial occlusion. In a model of cerebral vasospasm induced by autologous blood superfusion, PY 108–068 reversed the spastic state of cortical microvessels. In a chronic stroke model (microsphere embolization and its implication in a variety of pathological processes related to vascular diseases has recently become a major area of research. Calcium is considered to be involved in a variety of biochemical processes leading to contraction of smooth muscle cells, inhibition of mitochondrial respiration and activation of phospholipases with a concomitant increase in the concentration of free fatty acids and prostaglandin synthesis.

Due to the growing importance attributed to calcium in physiology and biochemistry, efforts have also been made to develop drugs able to interfere with the actions of calcium. Among the various drugs having been claimed to counteract the disturbances in calcium homeostasis, dihydropyridines belong to the most potent of those. We describe here the beneficial effects of PY 108–068, a potent calcium antagonist of the dihydropyridine type, on the protective properties of cerebral vessels.

The calcium antagonistic effect of PY 108–068 was investigated on isolated ring segments (n = 4–5) of the cat middle cerebral artery, according to a modification of the protocol by Edvinsson et al. The ring segments (length: 4 mm, diameter: 0.2–0.3 mm) were immersed in a depolarizing oxycarbon-saturated Krebs medium of following composition: mM NaCl = 65, KH2PO4 = 1.2, NaHCO3 = 25, glucose = 1, pH = 7.4. The rings were strained with a force of 500 mg. PY 108–068 was added to the bath at a dosage of 10−9 M. The isometric force which developed was registered on a Texas Instrument

EFFECT OF ANTI-OXIDANTS AND PFC ON CEREBRAL ISCHEMIA/Suzuki et al

recorder. The dose-response curve was then registered and pA2 values calculated as defined by van Rossum.22

2. Acute Experiments in Cats

Acute experiments were performed on cats, anesthetized with N2O/O2 (70%, 30% v/v) and artificially ventilated after paralysis with Flaxedil. Arterial blood pressure, blood gases, heart rate and end-expiratory pCO2 were monitored. Body temperature was kept constant at 37°C. Cranial windows were opened via trepanation in the parietal region and the dura was carefully removed. Tissue PO2 measurements were performed polarographically via a surface microelectrode which consisted of a glass-embedded platinum wire (tip diameter of 2–5 \( \mu \text{m} \)) and a silver silver chloride reference electrode placed in its immediate vicinity. Tissue PO2 measurements were performed by moving the electrode over the ischemic area to allow recording a large number of individual values. These were then grouped and plotted as a typical distribution histogram. The electrodes were calibrated before and after each experiment as well as between the essential episodes of the experiment. The technique was applied to following models of cerebral ischemia:

a: Focal, Epicerebral Ischemia

Focal epicerebral ischemia was induced in the cortex of cats by clamping a surface branch of the MCA in the ectosylvian gyrus with a specially sharpened neurosurgical microclip (n = 6). This technique allowed the occlusion of vessels as small as 200 \( \mu \text{m} \) diameter, which resulted in ischemic foci of approximately 10–20 mm^2. Tissue PO2 measurements were performed as described above. The numerous individual values were plotted on a histogram, of which the 95% confidence interval for the median was established. Statistical analysis was performed by using the U-test which compares two independent samples (non parametric counterpart to the t-test). Tissue oxygen distribution after treatment was compared with values before treatment. The drug PY 108–068 was administered orally once a day over a period of 3 days at a dosage of 300 \( \mu \text{g/kg} \) in 13 animals. The first drug application occurred 90 min after embolization, because histological analysis of rat brains had shown that severe changes such as edema, necrosis or leucocyte infiltration occurred by 2 hours after insult. Control animals received the same volume of the vehicle. Every morning, a neurological evaluation was made for each rat, consisting of the observation of the following possible spontaneous symptoms and of the establishment of a score which corresponds to the degree of severity:

<table>
<thead>
<tr>
<th>Score</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no symptoms</td>
</tr>
<tr>
<td>+</td>
<td>mild ataxia</td>
</tr>
<tr>
<td></td>
<td>or monteral rotation</td>
</tr>
<tr>
<td></td>
<td>or contralateral head tilting</td>
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<tr>
<td></td>
<td>or piloerection</td>
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<tr>
<td></td>
<td>or elevation of tail</td>
</tr>
<tr>
<td></td>
<td>or hyperirritability</td>
</tr>
<tr>
<td>+ +</td>
<td>severe ataxia</td>
</tr>
<tr>
<td></td>
<td>or severe contralateral rotation</td>
</tr>
<tr>
<td></td>
<td>or contralateral body tilting</td>
</tr>
<tr>
<td>+ + + +</td>
<td>contralateral hemiplegia</td>
</tr>
<tr>
<td>+ + + + +</td>
<td>death</td>
</tr>
</tbody>
</table>

The evaluator had no knowledge of which animals had received the drug or the vehicle. If several symptoms were associated, then the final score consisted of the addition of the individual notations. If no death occurred, the maximal possible score per animal was fixed to 6.

Neurological scores were compared statistically in...
Effect of PY 108-068 (10^{-9} M) on the concentration-response curve to CaCl$_2$ in the isolated cat cerebra media artery.

FIGURE 1. Effect of PY 108-068 (10^{-9} M) on the concentration-response curve of feline middle cerebral artery rings contracted by CaCl$_2$ loading (n=4).

Both treated and untreated groups by using the Fisher test for non-parametric samples. At the end of the experiment, the brains were removed. After verifying that the microspheres were lodged ipsilaterally, the brains were weighed and placed into a drying oven at 95°C for 4 hours. Brain water content was then calculated according to the dry/wet weight method.

Results

1. Isolated Brain Vessels in Vitro

The ring segments contracted in a dosage-dependent manner on addition of CaCl$_2$. Figure 1 shows the concentration-response curve of the effect of CaCl$_2$ before and 15 min after addition of 10^{-9} M PY 108-068. The shift to the right of the concentration response curve demonstrates the competitive inhibition of the effect of calcium by the drug. The pA$_2$ value is 10 ± 0.2 (± SEM).

2. Focal Epicerebral Ischemia

Figure 2a illustrates the effect of arterial occlusion on tissue oxygenation in the focal ischemic area of control, vehicle-treated cats. There is a mean decrease in pO$_2$ on the order of 30% following clamping of the feeding vessel. After a few minutes one observes a stabilization of the oxygen supply on this level.

Treatment with PY 108-068 leads to a progressive, significant increase in O$_2$ supply within the infarcted area. Simultaneous recording of the systemic blood pressure shows that after an initial decrease, this parameter is not further influenced by the drug infusion (fig. 2b).

3. Cerebral Vasospasm

Induction of vasospasm by cortical superfusion of hemolyzed blood leads to a marked decrease in tissue oxygenation (fig. 3). The pO$_2$ histogram is shifted to the left, due to an accumulation of low values with concomitant disappearance of high values. Such a hypoxic state is indicative of marked arterial vasoconstriction of the cortical vessels. Determination of capillary diameter at the end of the ischemic period confirms that also the microvessels are constricted (fig. 4). In the lower panel of figure 3, it can be seen that PY 108-068 not only completely normalizes the pO$_2$ histogram but even shifts it further to the right. Measurement of capillary diameter after the treatment shows that reopening of the microvessels is correlated with the observed normalization of tissue oxygenation (fig. 4).

4. Chronic Hemispheric Multiembolization

The rats recovered from anesthesia some minutes after operation. However, the first motor disturbances were only visible some 4–5 hours after embolization. After 24 hrs of chronic infarction, the mortality rate in the control group was already 30%, whereas no animal treated with PY 108-068 had died (fig. 5, upper panel). The surviving animals exhibited one or several of following symptoms: ataxia, hemiplegia, contralateral rotating, tilting of head or body, hyperirritability, etc. The mean neurological score in this group was significantly lower than in surviving control rats (fig. 5, lower panel). There was a transient increase in the score of the treated group on the second day, due to the...
FIGURE 2b. Treatment with continuous infusion of PY 108-068 (2 μg/kg/min) in 6 cats over 3 hours leads to a significant increase in O2 supply to the ischemic tissue as compared with the ischemic values (*p < 0.05, **p < 0.025, U-test). The lower panel shows the variation in mean arterial blood pressure during the experiment.

inclusion of died animals with a high score. Then, the score in both groups declined progressively because of spontaneous recovery. After 3 days, 50% of the control rats had died, the mortality being only 23% in the treated group. Established for the maximal score level of 7, the statistical analysis showed that the difference between both groups was significant at p < 0.016 on day 1, p < 0.008 on the second day and p < 0.007 on day 3.

In spite of a trend towards reduction, the development of brain edema was the same in both control and treated animals, as indicated by the brain water content in both embolized and non-embolized hemispheres (fig. 6).

Discussion
The calcium antagonistic properties of PY 108-068 are verified in experiments using isolated arterial rings. By placing the segments in a depolarizing Krebs-medium, neurotransmitters which could influence the vasomotricity of the preparation are depleted from receptors located in the vessel wall. Thereby, the relaxant effect of PY 108-068 on vessels rings contracted by calcium loading can specifically be attributed to a calcium-blocking property of the drug.

Epicerebral arterial occlusion by means of a microclip can be considered to be a variation of middle cerebral artery occlusion (MCAO). It has been shown by Ravvin et al.\textsuperscript{10} that occlusion of a major Sylvian branch of the MCA leads to a 25–30% reduction in CBF in the central i.e., the most ischemic area. In MCAO experiments, Bremer et al.\textsuperscript{3} found a pO2 reduction of 30% and Ingvar et al.\textsuperscript{9} reported a 40% decrease in tissue oxygenation. These data correlate well with

FIGURE 3. Effect of autologous, hemolyzed blood superfusion on cortical tissue pO2 distribution in 6 cats (upper and middle panels). In the lower panel, the antivasospastic effect of PY 108-068 is illustrated by the normalization of the histogram. Arrows indicate the center of gravity of the histograms.

FIGURE 4. Distribution histogram of capillary diameters in the cerebral cortex before and during vasospasm and after treatment with PY 108-068. Both control and posttreatment histograms are super-imposed, illustrating the anticonstrictive effect of PY 108-068 on microvessels rendered spastic by autologous blood superfusion.
our findings of a 35% reduction in oxygen pressure after epicerebral occlusion. It is of interest to note that, in contrast to ischemic situations such as generalized oligemia or vasospasm, there are much fewer low pO2 values in the tissue in this model,24 which indicates that the collateral circulation is able to compensate to some extent, thus avoiding the appearance of extreme hypoxic zones. Therefore, our model appears to provide identical microvascular effects as are observed in MCAO experiments. Further, excellent reproducibility is obtained and the technique is more easily performed. It has been shown that small cortical vessels such as minor arterioles and capillaries constrict after arterial occlusion,14-16 probably as a consequence of perfusion pressure fall. Measurement of ions in cortical tissue shows that MCAO leads to a marked decrease in extracellular calcium.14 Brandt et al1 have shown that the arteriolar constriction which follows MCAO can transiently be abolished by perivascular application of the calcium antagonist Nifedipine. Moreover, beneficial effects of the calcium antagonist Flunarizine are described in cases of ischemic capillary-opathies in the retina.13 Our data show that, in spite of a moderate decrease in systemic blood pressure, continuous infusion of PY 108-068 increases the oxygen availability of the ischemic core. Whether the beneficial effect of PY 108-068 on the cortical microcirculation is due to an alleviation of postischemic spasms in the ischemic focus or results from an interference of the drug with adrenergic receptors mediating vasoconstriction20,21 cannot be determined.

Superfusion of the brain with serum or hemolyzed blood is a good model for acute studies on cerebral arterial spasm, the model aiming at mimicking subarachnoid hemorrhage.1 It allows reproducible and rapid induction of cortical vasospasms which also affect the microcirculation.17,18 In previous studies performed with this model, we have shown that the cortical microcirculation is highly compromised by this procedure.24,25 Similar data have been provided by Hart.5 Comparing the effects of this type of ischemia with those occurring under oligemic conditions, we have found identical results concerning pO2 distribution or capillary morphometric dimensions.25 By comparing the effects of alphalytic drugs or of an elevation in systemic blood pressure in both models, we showed that the capillary constriction is reversible in oligemia but not in spastic ischemia, which led us to propose the hypothesis that capillaries can actively constrict.27

**Figure 5.** The upper panel shows the cumulative mortality over 3 days in control (black columns) and PY 108-068-treated (white columns) rats suffering from subchronic brain ischemia induced by microsphere embolization. The lower panel shows the mean neurological score (x ± SD) and its evolution over 3 days in embolized control rats (full line) and in rats treated daily with 300 μg/kg PY 108-068 per os (dotted line).

**Figure 6.** Brain water content (x ± so) in the embolized, left hemisphere and in the non-embolized, right hemisphere at the end of a 3 day period of microsphere embolization in control (black column) and PY 108-068-treated rats (white column).
Therefore, it is conceivable that calcium is involved in the blood-mediated vasoconstriction and this hypothesis has been confirmed by measuring the calcium activity in a model of SAH. Hubschmann et al. have shown that extracellular calcium is diminished while potassium accumulates. The data we report here confirm the in vitro studies on isolated brain arteries i.e., spastic vessels can be relaxed by PY 108-068 after calcium has accumulated. The morphometric analysis shows a complete normalization of capillary diameters. The shift to the right in the pO2 distribution histogram not only indicates that the spastic state of cortical vessels has been completely alleviated but also that the drug makes available more capillaries to perfusion, thus augmenting the oxygen partial pressure of brain tissue. This could be due to relaxation of precapillary sphincters which regulate the distribution of microflow. In a parallel study, we have observed that PY 108-068 continues to reverse spasms at a time point when vasodilators or adrenergic blockers are ineffective (unpublished observations).

Quantitative microembolization performed by means of 50 μm microspheres is one of a few possible models of "chronic" brain ischemia, allowing the observation of surviving animals over a few days. By using the retrograde injection technique into the internal carotid artery, the emboli lodge almost exclusively in the ipsilateral hemisphere and lead to ischemia in preferential structures such as cortex, hippocampus and striatum. The unilateral nature of the infarction permitted observation of neurologic disorders confined to the contralateral side (ataxia, rotations, tilting, hemiplegia). By varying the size and the amount of microspheres, the severity of the injury can be adjusted so that only a fraction of the animals die. Therefore, one can establish a mortality rate which is used as a criterion for drug effects. During the first 2 hours after embolization, the animals usually show only sedation. Histological and morphological analyses performed in our laboratories have shown that evidence for edema, inflammatory processes or cell necrosis can be seen from 2 hours postinfarction. Therefore, we selected 90 min postinfarction to be the time of first therapeutic intervention i.e., when lesions were thought to be still reversible. After this period, spontaneous motor deficits appear in the survivors, which can be followed over a period of days. The symptomatology of these animals is weighed according to a numerical score. A further advantage of such a model is the possibility of oral drug application, which provides important indications about the drug’s bioavailability. Our results show that the mortality is strongly reduced in animals treated with PY 108-068 and that the symptomatology is also significantly attenuated when compared with the vehicle-treated group. We clearly cannot distinguish whether rats die from cerebral or cardiac death. However, we never were able to find any microspheres in other organs than brain, suggesting that if rats died from cardiac failure, this was due to cerebral ischemia and to brain-heart relationships.

The protection afforded by PY 108-068 against neurological symptoms is clearly not due to an antiedematous effect. In other laboratories, it has been demonstrated that edema, which is a major complication in this model, affects cognitive functions such as learning ability or memory rather than motor disturbances. The anti-ischemic effect of PY 108-068 in such a model is an important contribution to the study of the drug in cerebrovascular disorders because it provides demonstration of the drug’s efficacy when administered orally and after induction of stroke (posttreatment).

Partial oxygen pressure measurements (pO2), an excellent indicator of microvascular changes, have demonstrated the protective effect of PY 108-068 against vascular disturbances in the brain. The drug proved to be beneficial in various types of ischemic insult (low flow, spasm, multifractiion). Furthermore, PY 108-068 proved to be remarkably efficacious in a severe ischemic situation (multifocal embolization) after peroral administration in low doses. As PY 108-068 also has particularly favorable hemodynamic properties when compared with other dihydropyridines this drug appears to be very promising for the treatment of cerebrovascular accidents.

References
Calcium antagonist PY 108-068: demonstration of its efficacy in various types of experimental brain ischemia.
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