Severe vasospasm was noted in the arteries and arterioles. An increasing number of degenerative changes were seen in the adrenergic nerves 8 and especially 16 h after freezing (fig. 2). This led to the total disappearance of the catecholamines from the central ischaemis area 24 h after freezing. Moreover, no mast cell fluorescence was seen in this area. The accumulation of catecholamines in nerve trunks at the margin of the injured area was also clearly apparent. In the present study, the disappearance of 5-HT fluorescence from the mast cells of the skin and the degranulation of mast cells were observed after experimental skin freezing. Since 5-HT and histamine are located in the same granules of rat mast cells[13], it may be assumed that both these granular components are liberated following the present experimental cold injury. The present observations provide morphological evidence corroborating Lewis's[14] theories concerning the possible role played by histamine-like substances in cold injuries.

The catecholamines liberated from degenerating adrenergic nerves provide a considerable stimulus to the effector cells[15]. The catecholamines liberated from the degenerating adrenergic nerves in frozen skin during the 1st 24 h after thawing, as observed in the present study, may thus increase the vasospasm of the injured tissue. The vasospasm in the cold injured tissue might further be increased by catecholamines accumulating in the adrenergic nerves at the margins of the cold-injured area. The present skin-freezing experiment leads us to emphasize the important role of mast cells and andenergic nerves in cold injury. The freezing method adopted for the present study is commonly used for tissue destruction in cryosurgery. In slow freezing lesions caused by clinical cold injury, icecrystals are formed predominantly extracellularly[1].

Further studies are needed to evaluate whether slow freesing also causes secondary effects on adrenergic nerves and mast cells. The following suggestions are made to stimulate further experimental and clinical testing.

Hypothesis. It is proposed that in addition to direct cell injury and injury to the vascular wall, vasoactive substances liberated from the mast cells may also in part be responsible for the increase in blood vessel permeability beginning during the acute phase following freezing injury. During the 1st 24 h after freezing of the skin, the catecholamines liberated from the degenerating adrenergic nerves of the frozen skin, as well as those catecholamines beginning to accumulate at the margin of the cold-injured area, may further increase the vasospasm and thus diminish the blood flow in the injured area.

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Sensitivity of follicular melanoblasts in newborn mouse skin to tritiated thymidine: Evidence for a long term retention of label[1]

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Summary. Injection of tritiated thymidine into newborn mice results in a progressive greying of hair that does not begin until after the first hair coat is grown. After a year the depigmentation is appreciable (about 60% of the hair are white). The effect cannot be simulated by external irradiation of newborn mice or by the administration of radioactive uridine or methionine. The effect can best be explained by a long-term retention of radioactivity in the DNA of melanocyte stem cells (melanoblasts) in spite of several rounds of cell division. This could be achieved by labelling the strands of DNA destined to act as templates throughout life by being selectively retained in the stem line as described in Cairns' hypothesis.

Cairns[2] has recently suggested that stem cell populations might have evolved a mechanism for selectively segregating the new from the old DNA strands at division. If the older, template, strands were conserved in the stem line this would protect the stem cells from DNA replication-induced errors. The evidence in support of this hypothesis has been reviewed elsewhere[2-5]. If the template strands of DNA in stem cells were labeled at the time when they were undergoing their terminal developmental cell divisions (e.g. in the skin of a newborn mouse) then cells might persist as labeled cells for a long time since they would not halve their radioactivity at each successive division. The only way in which the radioactivity might be reduced is via sister chromatid exchanges. A selective retention of a 'permanently' labeled complement of DNA strands (chromatids) has been suggested as the explanation for the persistence of labeled cells within the stem cell region of crypts of the small intestine that were labeled during post-irradiation regeneration[6].

I present here a series of experiments where newborn mice were labeled with tritiated thymidine (^{3}HTdR) which was assumed to be incorporated into the stem cells for the hair follicle pigments cells, the melanoblasts, during their establishment in newly forming hair follicles. These melanocyte precursors gradually died over a period of 1-3 years suggesting that the label was retained over the many cell generations required for the successive hair growth cycles over this period i.e. observations in accord with the Cairns' hypothesis.

Hair follicles are formed by downgrowths from the epider-

mis which in rodent skin occur in the interval from a few days before birth to a few days after birth. This process leads to the establishment of the full adult complement of hair follicles[7] each of which contains the stem cells for the hair follicle matrix and the stem cells (melanoblasts) for the pigment forming cells, the melanocytes. Once formed these structures represent closed units of proliferation that undergo cyclic phases of hair growth and quiescence. Each resting follicle is believed to contain only 1 or 2 melanoblasts[8] which divide during the early stages of hair growth to provide the 15-30 melanocytes[9]. Unfortunately, it is not known for sure whether the melanoblasts divide once to replace themselves and produce a daughter that divides further to produce the melanocytes, or whether the melanoblasts undergo 3-5 divisions at each hair cycle with 1 or 2 cells retained as melanoblasts for the next cycle (see fig. 1). The latter has been suggested[10] but proof is largely lacking and the present data conflict with this idea.

If the melanoblast population is destroyed, for example by radiation, the surviving follicles will be permanently depigmented[8-13]. When adult mouse skin containing resting hair follicles (with resting melanoblasts) is X-irradiated the melanoblast survival curve has a radiosensitivity that is characterized by a D_0 value of about 2 Gy (a measure of the slope of the exponential part of the survival curve plotted on a semilogarithmic plot). This clonal survival shows resting melanoblasts to be slightly more resistant than, or similar to, many other in vivo cell systems assayed using cell survival curves[14-21]. The radiosensitivity of the melanoblasts in growing hair follicles is even lower, i.e., they have greater resistance[21].

Baserga and coworkers[22,23] observed that doses (e.g. about 10 μCi/g) of tritium labeled thymidine (^{3}HTdR) given to newborn brown mice resulted in a shortening of lifespan and an increase in tumor yield. Calculation of the radiation dose is difficult and controversial but the effects observed most probably reflect radiation damage to the DNA in the various stem cell populations in newborn animals. Carcinogenic events must be initiated during the 1st week of life since the thymidine must be diluted rapidly by cell division. These fixed initiation events must then require further changes over the life of the animal before expression as cancerous growth. The shortening in lifespan might represent some defect, also induced during early life, affecting the number or long-term proliferative potential of stem cell populations. Label will also be incorporated into cells destined for the slowly replacing or non-replacing tissues (e.g. connective tissue) within which the eventual accumulated radiation dose (and damage) must be considerable and this could account in part for the effects observed.

In the present experiments B6D2F1 (C57 Bl/6J ♀ × DBA/2J ♂) newborn animals were used. These have a uniform black coat when adult but are heterozygous for black (Bb) (fig. 2). The newborn mice received 5 μCi of tritiated-6-thymidine (^{3}HTdR) (~ 3.3 μCi/g) s.c. in the center of the back twice daily for 3 days after birth (at 09.00 and 17.00 h), i.e., a total dose of 30 μCi (~ 20 μCi/g) over 3 days (sp. act. ~ 30 Ci/mM). Other newborn mice received 5 μCi of 5,6 ^{3}H-uridine (^{3}HUR), 5 μCi L-methyl ^{3}H-methionine (^{3}H-meth), various doses of γ rays (^{137}Cs, 5 Gy/min) or 0.25 mg bromodeoxyuride (BUdR) twice daily for 3 days. In the latter case these animals were kept in the dark until 4 weeks

Table 1. The proportion of white to fully-pigmented hair in B6D2F1 mice labeled when newborn

Treatment	Age (days)	White: total	%	White: total zigzags	%	White: total guard	%	Number of mice
Saline	365	0: 557	0	0:372	0	0:185	0	3
	695	0: 437	0	0:321	0	0:116	0	3
	1093	0: 372	0	0:272	0	0:100	0	1
Thymidine	610	16: 608	2.6	13:423	3.1	3:185	1.6	4
^{3}HUR	84	4: 378	1.0	4:224	1.8	0:154	0	2
	365	58: 695	8.3	54:454	11.9	4:241	1.7	4
^{3}H Methionine	84	0: 190	0	0: 84	0	0:106	0	1
	309	6: 669	1.0	3:407	0.7	3:262	1.1	4
^{3}HTdR	56	78: 870	9.0	55:384	14.3	23:486	4.7	4
	84	73: 872	8.4	51:448	11.4	22:424	5.2	4
	134	180: 594	30.3	138:398	34.7	42:196	21.4	3
	252	162: 608	26.6	150:420	36.0	12:188	6.0	4
	364	593:1004	59.1	488:617	79.1	105:387	27.1	5
	675	654: 874	74.8	581:659	88.0	73:215	34.0	5
	1094	506: 873	58.0	426:630	67.6	80:243	32.9	2

Table 2. The proportion of white to fully pigmented hair in B6D2F1 mice labeled when newborn. ^{137}Cs ray experiments

Treatment	Age (days)	White: total	%	White: total zigzags	%	White: total guard	%	Number of mice
0.21 Gy	56	0:506	0	-	-	-	-	4
daily for 10 days	84	0:493	0	-	-	-	-	4
	702	1:307	0.3	1:201	0.5	1:106	0.9	2
0.21 Gy daily for 3 days	56	0:485	0	0:214	0	0:271	0	2
0.74 Gy once	56	0:481	0	-	-	-	-	2
0.5 Gy twice daily for 3 days	470	15:363	4.1	8:194	4.1	7:169	4.1	2
0.98 Gy once	56	0:376	0	-	-	-	-	2
5 Gy once	84	0:188	0	0:100	0	0: 88	0	1
	458	19:340	5.6	12:222	5.4	7:118	5.9	2
0.44 Gy in utero	56	0:404	0	-	-	-	-	2

of age when they were plucked[9,24] and irradiated with UV-light (2 or 4 min exposure of 4 cm^2 to a 250 W high pressure mercury vapour lamp with a quartz window and a Corning 9863 filter which produced a beam with a spectrum of 250–410 nm and an energy deposition of about 2×10^6 erg/min/cm^2). A few 7–8-week-old mice received 100 µCi ^{3}HTdR or were plucked and about 18 h later received 100 µCi ^{3}HTdR or saline twice daily for 3 days. Control litters received a series of s.c. saline injections or 0.05 µg of non-radioactive thymidine twice daily for 3 days.

The results are presented in tables 1–4. In the control (saline injected) animals no white hair were observed even in 3-year-old animals (table 1). However, an effect was detectable even in the first pelage of those animals that received ^{3}HTdR. There was then a progressive increase in white hair up to an age of about 1 year by which time a possible plateau level of about 60% white hair is reached (table 1; fig. 2). The proportion of white underfur (zigzag) hair[25] is higher (about 70–80%) which is probably a consequence of the fact that these are the smaller follicles presumably with fewer melanoblasts. This suggests that the ^{3}HTdR injected into neonates is retained for at least a year in the melanoblast stem cells. The greying occurs mostly in the regions overlying the original site of injection. There is a slight reduction in body weight in the ^{3}HTdR treated animals at an age of 1 year, 24.1 ± 0.4 g for treated vs 27.0 ± 0.4 g for controls. ^{3}H-uridine induces a few white hair while ^{3}H-methionine hardly any at all. Non-radioactive thymidine does not induce any appreciable greying (table 1). The ^{3}HTdR effect cannot be simulated by doses of external γ-rays up to single doses of 5 Gy (table 2) indicating that it cannot be an acute radiation effect. Bromodeoxyuridine incorporated into neonates persists in sufficient quantities such that the BUdR containing DNA is sensitive to UV-rays when delivered 4 weeks later (table 3). Within the 4 cm^2 UV-irradiated area 60% of the underfur hair is depigmented: an effect not seen after either UV, alone, or BUdR alone indicating that the UV must reach the level of the target cells. Thus, DNA labeled in the neonate must remain largely undiluted for at least 4 weeks. The administration of ^{3}HTdR to resting hair follicles (in 7–8-week-old mice), which have been stimulated into activity by plucking, results in an age-progressive greying of the pelage (table 4).

The major features of these experiments are as follows:

1. The proliferation capacity of hair follicle melanoblasts is sensitive to some action of ^{3}HTdR (although the possibility that differentiation expression (melanogenesis) is somehow inhibited cannot be entirely ruled out). 2. This effect does not appear to be due to short-term radiation-induced damage since it cannot be simulated by external irradiation. 3. It appears to build up progressively over the period of a year. 4. The effect appears to be associated with DNA in that other labeled precursors do not produce any depigmentation. 5. The effect can be achieved by ^{3}HTdR administration whenever melanoblasts are undergoing replication, i.e., shortly after birth or after plucking adult hair.

The damaging effects of tritiated thymidine are extensively documented[22,23,26–33]. These effects range from relatively subtle changes in cell cycle behavior to impairment of reproductive activity and overt cell killing.

The present observations suggest that the hair follicle melanoblast (stem cells) may be sensitive at an early stage in skin hair follicle formation. Most of the melanoblasts are capable of forming the abnormal melanocyte complement for the first growing hair follicle. There is a subsequent progressive decline in hair follicle melanocyte repopulating (clonal) ability with subsequent hair cycles. After about a year 80% of the smaller zigzag follicles lack functional melanocytes. Hence, the damage builds up over a period of a year which suggests that the tritiated thymidine is retained in melanoblasts at damaging concentrations for at least a year. If higher doses of ^{3}HTdR are used an effect can be observed earlier (e.g. with 25 µCi 31.5% of the hairs are white by 84 days: 1298 white: 4119 total). Over a year a mouse will grow at least 4 hair coats, probably more (say

Table 3. The proportion of white to fully pigmented hair in B6D2F1 mice labeled when newborn. Bromodeoxyuridine experiments

Treatment	Age (days)	White: total	%	White: total zigzags	%	White: total guard	%	Number of mice
BUdR								
4-week Pluck	56	79: 472	16.7	41:134	30.6	38:338	11.2	3
UV 4 min	84	171: 561	30.5	135:225	60.0	36:336	10.7	3
Saline								
4-week Pluck	56	49:1150	4.3	11:312	3.5	38:838	4.5	8
UV 2–4 min	84	43:1468	2.9	19:638	3.0	24:830	2.9	8
BUdR								
4-week Pluck	56	14: 309	4.5	14:135	10.4	0:160	0	2
	84	26: 386	6.7	26:208	12.4	0:178	0	2

Table 4. The proportion of white to fully pigmented hair in 7–8-week-old B6D2F1 mice

Treatment	Time after treatment	White: total	%	White: total zigzags	%	White: total guard	%	Number of mice
Saline	56	0:556	0		0			2
	257	2:300	0.7	2:210	0.95	0:90	0	2
100 µCi ^{3}HTdR	56	0:267	0	-	0	-	0	2
	112	0:346	0	-	0	-	0	2
	257	0:320	0	-	0	-	0	2
Pluck wait 18 h	56	34:364	9.3	27:229	11.8	7:135	5.2	2
100 µCi ^{3}HTdR	112	104:458	22.7	52:266	19.5	52:192	27.1	2
	257	291:418	69.6	200:286	69.9	91:132	68.9	2
Pluck wait 18 h	56	14:360	3.9	7:151	4.6	7:209	3.3	2
saline	112	1:301	0.3	0:208	0	1: 93	1.1	2
	257	2:311	0.6	0:206	0	2:105	1.9	2

All injections twice daily for 3 days.

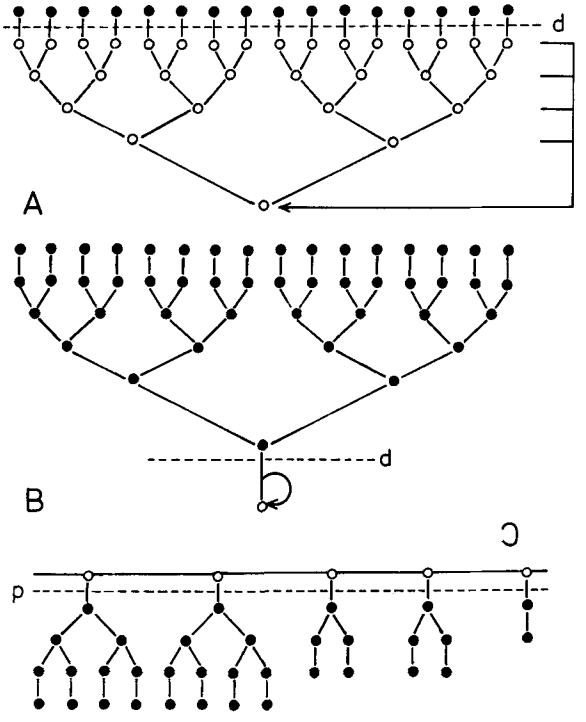

Figure 1. Possible schemes for replacement of follicular melanocytes during each hair growth cycle. Open circles, stem cells (melanoblasts); closed circles, differentiated (melanogenic) melanocytes; d, differentiation (dashed line).
A Model proposed in 1972[10]. The melanoblasts divide to produce melanocytes but only 1 or 2 persist through to the next hair cycle. Each melanoblast will divide up to 4–5 times. *B* Model proposed here. Melanoblasts divide once or twice to maintain themselves and produce differentiated progeny that divide further. *C* An intermediate model. Melanoblasts divide from 1 (model B) to 16 times producing differentiated progeny at each division. The cell division capacity of the maturing melanocytes might be variable (from 1 to 4, 4 = model B).
Functional melanocytes can be seen in division[10] which rules out both model A in its strictest form and the extreme example of model C (left hand side) where the melanoblasts divide 16 times unless dedifferentiation from functional melanocyte to amelonotic melanoblast (stem) can occur. Model A can be ruled out on the basis of the present experiments since label might be expected to be districuted (diluted) throughout the melanoblasts. If there were a cell line that selectively retained the label this amounts to model C an extreme example of which is model B, which is favored. The number of melanoblasts per resting follicle is thought to be only 1–2[8]. Hence the number of melanoblast cell divisions per hair cycle would be within the range 1–8, most probably 1 or 2.

4–6) since 4 coats will grow within about 150 days[25] and the resting phase probably lengthens with each cycle. The mechanism of melanoblastmelanocyte replacement at each hair cycle is largely unknown (see fig. 1) but during the 4–6 hair growth cycles the melanoblasts must have undergone at least 4–6 cell cycles and probably no more than about 16 cell cycles (assuming a 2–5-fold amplification at each hair cycle: see fig. 1). Assuming a random segregation of DNA strands at each cell division, this would mean that any initial radioactivity would be diluted to between 6.25% and 0.0014% of the initial level i.e. with the possible exception of the lower minimum number of hair cycles, to negligible levels. Since damage is only observed after several hair cycles the label must be retained in the melanoblasts in spite of cell division activity i.e. as predicted by Cairns[2].

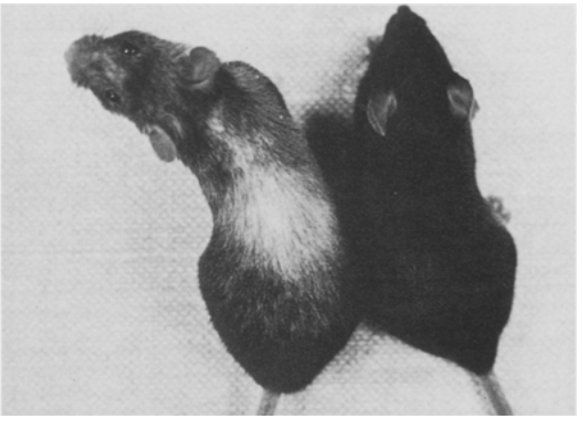

Figure 2. Photograph of 2 300-day-old mice. One received a series of saline injections at birth while the other received ^{3}HTdR. The appreciable greying (premature aging) is clearly evident.

These results would also permit the rejection of the earlier model for melanocyte replacement[10] where each melanoblast undergoes 3–4 divisions at each hair cycle and 1 or 2 of the cells are retained at the end of the cycle to act as stem cells for the next hair growth (fig. 1 A). Such a model would result in an even more rapid dilution of label than mentioned above which is incompatible with the present results. Each melanoblast stem cell must divide early in the hair growth cycle to provide one daughter that via further divisions produces the functional melanocytes while the other daughter remains as the stem cell for the next hair cycle (fig. 1 B).
The most likely explanation for the present results appears to be that the label incorporated into the newborn animals is retained in the melanoblast essentially throughout life in spite of at least 4–6 (probably more) cell divisions. This retained label eventually kills the melanoblasts and hence depigments the hair. This implies that the labeled DNA is selectively segregated at division into the daughter cell that is destined to act as the stem cell for the next hair cycle.

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Interaction of diltiazem with propranolol on atrioventricular conduction and refractoriness in the dog[1]

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Summary. Propranolol pretreatment (0.1 mg · kg^{-1}) significantly increased the lengthening induced by diltiazem (0.15 mg · kg^{-1} + 0.01 mg · kg^{-1} · min^{-1}, 20 min) of the A-H interval of His bundle potential recordings in the dog. In the presence of propranolol, diltiazem also significantly increased the atrioventricular effective refractory period. The results suggest the possible occurrence of AV blocks as a result of the use of a diltiazem-beta blocker combination in clinical practice.

Calcium blockers are sometimes used in combination with beta-adrenergic blockers to treat severe and unstable angina pectoris. Such combinations have been proved to be dangerous with verapamil[3,4], but seem much safer with nifedipine[5]. In the present study, the effects of the new calcium blocker diltiazem were assessed in the dog in normal conditions and after beta-adrenergic blockade by propranolol.

Materials and methods. 12 mongrel dogs weighing from 18 to 22 kg were anesthetized with choralose (80 mg · kg^{-1}) and were given dextromoramide 0.1 mg · kg^{-1} i.v. 10 min before control measurements. Dextromoramide was used to restore vagal tone[6] which was reduced by chloralose anesthesia[7]. The atrioventricular effective refractory period (AVERP) was then longer than the atrial ERP and could thus be measured. The animals were intubated and ventilated by means of a respirator (Bird MK VIII), using a mixture of air and oxygen. His bundle potentials were recorded under right atrium pacing at a constant rate, according to the technique of Sherlag[8]. Atrial, AV node and ventricular ERPs were determined by the extrastimulus method (for details, see Lièvre et al.[9]). Mean blood pressure was continuously recorded through a catheter percutaneously introduced into the right femoral artery. The body temperature was maintained between 38 and 39 °C by means of external heating.

The study comparing diltiazem alone and a diltiazem-propranolol combination was made after randomization of the animals into 2 groups. In 6 animals, diltiazem 0.15 mg · kg^{-1} was injected i.v. just after control measurements and simultaneously infused at the rate of 0.01 mg · kg^{-1} · min^{-1} for 30 min. In 6 dogs, propranolol 0.1 mg · kg^{-1} was administered 10 min before control measurements, followed by diltiazem administration. Measurements were performed before (control) and 10, 20, 30 min after the beginning of diltiazem administration. In each series, comparisons of means with the control state were made by Student's t-test for paired values. Changes in parameters from control values were compared between the 2 series by Student's t-test for unpaired values after a comparison of variances had proved it possible.

Results and discussion. Diltiazem alone had the same effects on AV conduction as other slow channel inhibitors[9,10] and increased A-H interval (i.e. conduction time through AV node). AVERP was not significantly increased (table). Mean blood pressure decreased slightly but significantly from 98.3 ± 11.6 to 88.3 ± 13.8 mm Hg ($p < 0.05$). There were no significant changes in S-A and HV intervals, heart rate, atrial and ventricular ERPs. Propranolol lengthened the A-H interval. The A-H interval control value was therefore higher in the diltiazem-propranolol combination series. The difference between control values of AVERP was not significant. Diltiazem-propranolol combination in-

Effects of diltiazem (0.15 mg · kg^{-1} + 0.01 mg · kg^{-1} · min^{-1} for 30 min) and diltiazem-propranolol combination (diltiazem, same dose, propranolol 0.1 mg · kg^{-1}, 10 min before control measurements) on A-H interval of His bundle potential recordings and atrioventricular effective refractory period (AVERP)

		Control	10 min	20 min	30 min
A-H	Diltiazem (n = 6)	51.3 ± 4.3	63.6 ± 7.1	63.6 ± 3.4[a]	64.6 ± 5.5[a]
(ms)	Diltiazem + P (n = 6)	92.0 ± 9.8	118.3 ± 7.9[b]	115.0 ± 8.8[c,d]	116.0 ± 8.1[c]
AVERP	Diltiazem (n = 6)	233.2 ± 37.5	241.6 ± 32.8	246.6 ± 27.8	235.0 ± 17.4
(ms)	Diltiazem + P (n = 6)	278.3 ± 19.9	331.6 ± 27.0[a]	348.3 ± 25.4[a,d]	355.0 ± 26.1[b,d]

Mean values and SEM before (control) and 10, 20, 30 min after the beginning of diltiazem administration. n, Number of experiments. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$: significantly different from control value (paired t-test); [d] $p < 0.05$: comparison of changes in parameters from their control values between the 2 series.