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Calcium release from frog sarcoplasmic reticulum by an imidazolyl reagent

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Summary. Calcium is released from the isolated heavy sarcoplasmic reticulum (SR) of frog skeletal muscle upon application of 0.1–1 mM diethylpyrocarbonate (DEP, an imidazolyl reagent). The Ca-ATPase activity of SR was suppressed by 20% in the presence of 1 mM DEP. More than 1 mM of free magnesium ion or 5 μM ruthenium red eliminated the effect of DEP on calcium release but not on Ca-ATPase activity. A plausible site of DEP action is on the calcium channel.
Key words. Fragmented sarcoplasmic reticulum; diethylpyrocarbonate; calcium release; Ca-ATPase.

Excitation-contraction coupling in skeletal muscle is accompanied by a rapid release of calcium from the sarcoplasmic reticulum (SR), in response to depolarization of the transverse tubular membrane. However, the investigation of molecular mechanisms involved in release of calcium from the SR requires further attention. Several methods are used to induce the release of calcium from the SR[1–3]. One is to chemically modify calcium channel proteins on the SR. Much attention has recently focused on the sulfhydryl group[4–7]. Sulfhydryl reagents appear to release calcium at lower concentrations than those that inhibit Ca-ATPase. On the other hand, other reagents, such as methionyl, imidazolyl, carboxyl, guanidyl or amino reagents can induce contraction of skinned fibers; an imidazolyl reagent, diethylpyrocarbonate (DEP), was most effective[8]. A clarification of the site of action would help in understanding the calcium release mechanism. We have now obtained evidence which suggests that DEP releases calcium by binding to the calcium release channel or to a regulatory structure of the channel of the heavy fraction of the SR, quite independently of the effect on Ca-ATPase.

Materials and methods

Fragmented heavy SR was prepared from leg muscle of *Rana catesbeiana* by the method of Koshita and Hotta[9]. The muscle was homogenized in a blender for 10 s in three volumes of 5 mM PIPES/Tris buffer (pH 6.8) 3 times with 40-s intervals, and then centrifuged for 1 h at 4000 × g. The supernatant was filtered through gauze and was centrifuged for 1 h at 10,000 × g. The pellet was suspended in 50 mM KCl, 20 mM Tris/maleate (pH 6.8), and recentrifuged for 10 min at 5000 × g. The pellet obtained by centrifugation for 60 min at 30,000 × g was stored in a small amount of 50 mM KCl, 20 mM Tris/maleate (pH 6.8) at − 70 °C. The protein concentration of the sample was determined by the Biuret method, standardized against bovine serum albumin.

Extravesicular calcium was monitored using a calcium selective electrode prepared by the method of Nakamura et al.[10], with some modification. A Pipetman polyethylene tip for 1–200 μl was dipped in the sensor cocktail (8.8% ETH1001, 0.88% sodium tetraphenylborate, 78.32% o-nitrophenyloctylether, 12% polyvinylchloride (n = 1000)). The sensor column was overlaid with an internal solution containing 100 mM KCl, 5 mM $CaCl_2$, 5 mM EGTA, 5 mM $MgCl_2$, and 20 mM MOPS (pH 7). The reference electrode of a glass pipette was filled with 1.5% agar in the internal solution, without $CaCl_2$ and EGTA. Electrical signals were fed to a high impedance differential amplifier (MEZ 7101, Nihon Kohden) and displayed on a conventional recorder. The calcium electrode gave a slope of 27–30 mV per pCa unit in a calibration solution containing calcium-EGTA between pCa 2 and 6.4 at experimental temperature (25–27 °C).

Solutions were sequentially poured into the chamber: 374 μl of base solution, 25 μl of 1 mM $CaCl_2$, 50 μl of 15 mg protein/ml SR, 6 μl of 0.5 M phosphoenolpyruvate (PEP), 5 μl of 1000 U pyruvate kinase, 20 μl of 0.1 M ATP, and 20 μl of several concentrations of DEP. The final concentration was 1.5 mg protein/ml SR,

100 mM KCl, 4 mM ATP, 6 mM PEP, 10 U pyruvate kinase, 20 mM MOPS, and 1 mM or 5 mM $MgCl_2$. Total calcium concentration was 97 ± 11 µM (mean ± SEM, n = 59), because of contaminating calcium in the SR sample. DEP was added to induce calcium release from the SR, 30 s after the addition of ATP. DEP did not affect the calcium binding to ATP or to PEP, since calcium concentration did not alter on the addition of DEP into the solution containing all components without SR. The solution was continuously stirred with a magnetic stirrer during calcium monitoring. Free magnesium ion was calculated as 0.04 mM or 1.4 mM in 1 mM or 5 mM total $MgCl_2$ solution, respectively. Five or six trials were done for each kind of tests. A control run without DEP was made for each SR sample.

The amount of calcium entering the SR (Ca_{SR}) can be calculated from total calcium (Ca_T), free calcium (Ca_{free}), and Ca-ATP:

$$Ca_{SR} = Ca_T - Ca_{free} - \text{Ca-ATP}$$

Ca-ATP is equal to Ca_{free} times Ca-binding capacity of ATP ($= K_{appCa/ATP} \cdot ATP_{free}$). ATP_{free} depends on magnesium content, ATP and calcium.

$$ATP_{free} = ATP_T - \text{Mg-ATP} - \text{Ca-ATP}$$

Therefore ATP_{free} also varies according to the level of Ca_{free}. However, when Ca_{free} is 1–3 µM, the following approximations can be used: if Mg_T = 1 mM, ATP_{free} is 3.00 mM and the binding capacity is 15.3 µM Ca-ATP per µM Ca_{free}; if Mg_T = 5 mM, these values are 0.26 mM and 1.32 µM per µM Ca_{free}, respectively. Sequestered Ca_{SR} is then:

$$Ca_{SR} = Ca_T - Ca_{free} \cdot (1 + K_{appCa/ATP} \cdot ATP_{free})$$

Released calcium ($\triangle Ca_{SR}$):

$$\triangle Ca_{SR} = - Ca_{free} \cdot (10^{\triangle E/slp} - 1) \cdot (1 + K_{appCa/ATP} \cdot ATP_{free}),$$

where $\triangle E$ means the difference of potentials (mV) (control minus test), and slp the slope of an electrode (mV) per pCa unit. Ca_{free} of control at the time of addition of DEP-blank solution was regarded as the same as the level in the test trace. Constants used were: $K_{appCa/ATP}$ = 5100, $K_{appMg/ATP}$ = 11 400.

Ca-ATPase activity was measured as the difference between the activity at pCa 7.3 and that at pCa 4.9. DEP was added to the SR (1.5 mg protein/ml) in a solution containing 100 mM KCl, 3 mM $MgCl_2$, 20 mM MOPS, 0.05 mM $CaCl_2$ and 0.5 mM or 0.03 mM EGTA (pH 7).

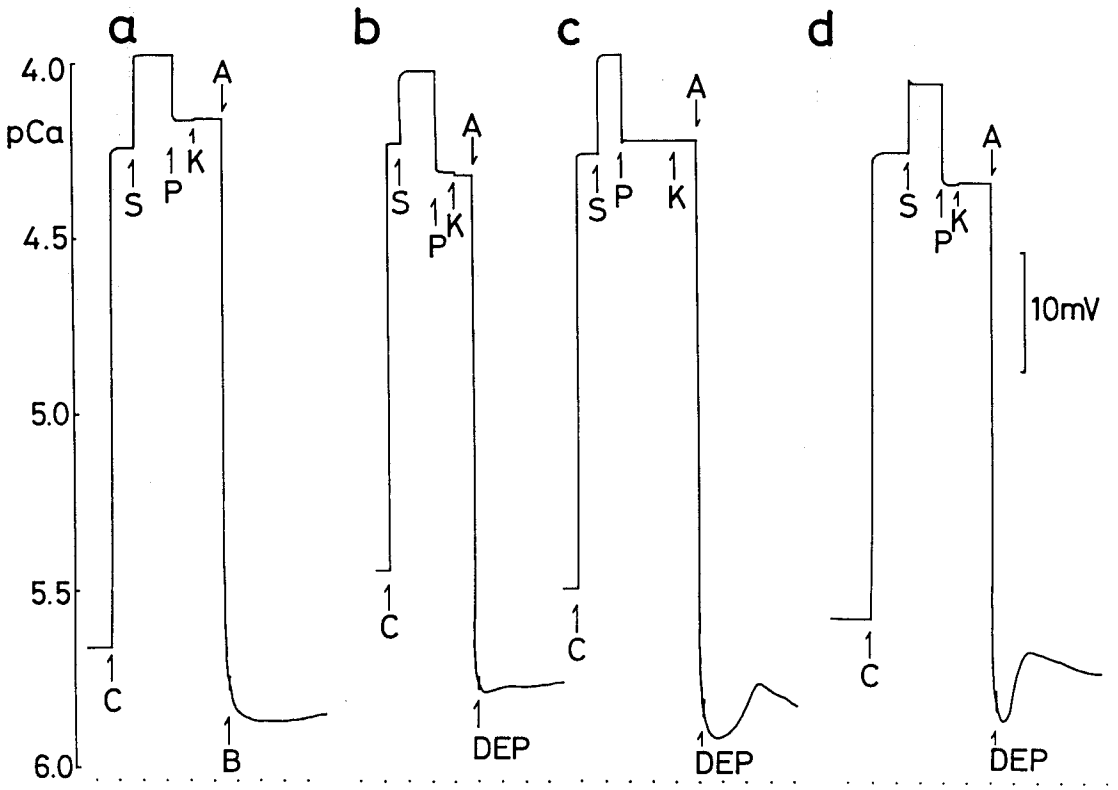

Figure 1. Calcium release from heavy SR, as induced by DEP. DEP concentrations (final) were 0 (*a*), 0.1 mM (*b*), 0.25 mM (*c*), and 0.9 mM (*d*). Several solutions were introduced into the chamber containing base solution (374 µl) at arrows: C, 1 mM $CaCl_2$ (25 µl); S, 15 mg/ml SR (50 µl); P, 0.5 M PEP (6 µl); K, 1000 U pyruvate kinase (5 µl); A, 0.1 M ATP (20 µl); B, blank solution of DEP (20 µl). Considering the dilution, final concentration of total calcium was 86 µM (*b*), 93 µM (*c*), and 81 µM (*d*). Ca_{free} at the peak of calcium release were 1.78 µM (*b*), 1.75 µM (*c*), and 2.19 µM (*d*). Slope of the electrode value was 29.6 mV per pCa unit. Dots under each trace represent the time mark (once 2 min).

Following incubation for 20 s to 3 min, the solution was mixed with 74 volumes of ATP solution (final: 100 mM KCl, 3 mM $MgCl_2$, 20 mM MOPS, 0.05 mM $CaCl_2$, 0.5 mM or 0.03 mM EGTA, 2 μM A23187, and 2 mM ATP, pH 7) to initiate the ATPase reaction. Aliquots were sampled 4 times at 30-s intervals and the reaction was halted using ice-cold 15% TCA. Inorganic phosphate in the solution was quantified by photometry[11,12]. When $MgCl_2$ was decreased to 1 mM, $CaCl_2$ was increased to 0.09 mM to give the same pCa (4.9) as that in 3 mM $MgCl_2$ solution. Free magnesium ion was 0.11 mM or 1.2 mM in 1 mM or 3 mM total $MgCl_2$ solution, respectively. Free ions were calculated by a computer with a modified program based on the one of Fabiato and Fabiato[14] (apparent Km for Mg/ATP:11 400, Mg/EGTA:40.5, Ca/ATP:5100, Ca/EGTA:2.51×10^6). Experiments were performed at 25–27 °C. The concentration of DEP was estimated after each experiment by the method of Ehrenberg et al.[13].

Results and discussion

a) Calcium release from SR by DEP. The effect of DEP on the SR was examined by directly monitoring the extravesicular concentration of calcium in the heavy SR, using a calcium electrode. About 1 mM DEP released calcium from the actively calcium-loaded SR (figs 1 and 2). The rate of release of calcium was faster in 1 mM DEP than 0.3 or 0.1 mM DEP, although the rate and amount released differed among the SR samples. The time required from application of 1 mM DEP to the peak of calcium concentration was between 40 and 175 s. In figures 1d and 2a, showing the effect with 0.9 mM DEP, Ca_{free} was 2.19 μM at the time of the calcium-release peak, a 7.4 mV increase from control (Ca_T = 80.7 μM); the released calcium is:

$$\begin{aligned}\Delta Ca_{SR} &= -Ca_{free} \cdot (10^{-7.4/29.6} - 1) \cdot 16.3 \\ &= 16\ \mu mol/l \\ &= 10\ nmol/mg\ protein \\ &= 25\%\ of\ Ca_{SR}\end{aligned}$$

Mean calcium taken up into the SR up to the peak of calcium release was estimated as 48 nmol/mg SR protein, and calcium released by 1 mM DEP was 9 μM (2–16 μM), 6 nmol/mg SR protein, 13% of taken-up calcium (n = 5). About 0.1 mM DEP did not induce the release of calcium in 2 out of 5 cases. DEP-induced calcium release was usually followed by a calcium reuptake, although the rate of uptake was often slower than the control. Compared with the reports from other laboratories[5,6], calcium release in the presence of DEP was less and slower than that induced by sulfhydryl reagents.

The DEP-induced calcium release was noted to have properties similar to caffeine-induced calcium release or calcium-induced calcium release. The calcium release induced by about 1 mM DEP in the presence of 1 mM $MgCl_2$ was blocked by either increasing the magnesium concentration or adding 5 μM ruthenium red (fig. 3a, b). The late and slow release of calcium was evident under these conditions. In 5 mM magnesium, 0.96 mM DEP (fig. 3a), released calcium at 5 min (Ca_{free} = 2.13 μM, Ca_T = 99 μM) is:

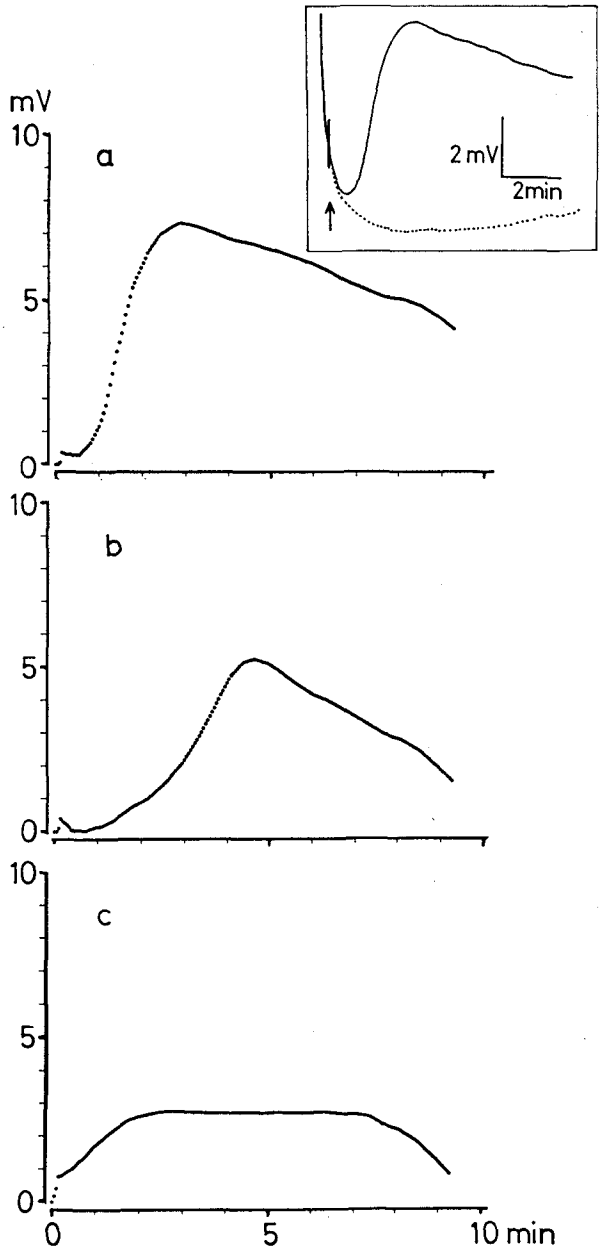

Figure 2. Subtraction traces of calcium release in figure 1. The inset shows an original record of extravesicular calcium concentration. Upward deflection indicates an increase in calcium concentration in the medium. 0.9 mM DEP was added at the arrow (30 s after ATP addition), a time at which the calcium uptake is not completed, as shown by downward deflection. Dotted line in the inset shows a control run. Subtraction of the control recording from the test recording by a computer gives trace a. DEP was added at time 0 on this trace. Traces b and c were calculated in the same way for 0.25 mM and 0.1 mM DEP, respectively. Peak value was 7.4 mV (*a*), 5.2 mV (*b*), and 2.7 mV (*c*). Released calcium in nmol/mg SR protein (% of taken-up calcium) were: (*a*) 10 (25%), (*b*) 6.3 (13%), (*c*) 3.7 (9%).

$$\triangle Ca_{SR} = -Ca_{free} \cdot (10^{-3.3/28.4} - 1) \cdot 2.32$$
$$= 1.2\ \mu M$$
$$= 0.8\ nmol/mg\ protein$$
$$= 1.2\%\ of\ Ca_{SR}$$

Released calcium at 10 min was 1.5 µM, 1 nmol/mg SR protein, 1.6% of the calcium taken up. As the peak of calcium release in the presence of DEP was not reached, the DEP-induced rapid release of calcium seems to be inhibited by magnesium and ruthenium red.

b) Inhibition of Ca-ATPase in SR by DEP. A high concentration of DEP slightly depressed Ca-ATPase activity (fig. 4a). Mean activity ± SEM was 2.73 ± 0.15 µmol Pi/mg SR protein/min in control and 2.20 ± 0.12 in 1 mM DEP for 1 min, thereby indicating about 20% inhibition of the Ca-ATPase activity by 1 mM DEP. The addition of 0.1 or 0.3 mM DEP to the medium led to no significant inhibition of the ATPase activity in the SR. The Ca-ATPase activity was transiently activated when measured 20 s after 1 mM DEP addition. Thereafter, the activity was inhibited and was maintained at a steady level (fig. 4b). Shoshan-Barmatz[15] reported briefly that DEP had no effect on Ca-ATPase activity and on calcium accumulation in rabbit fragmented SR vesicles. This discrepancy may arise from the different order of organism used (amphibian vs mammalian), and from differences in

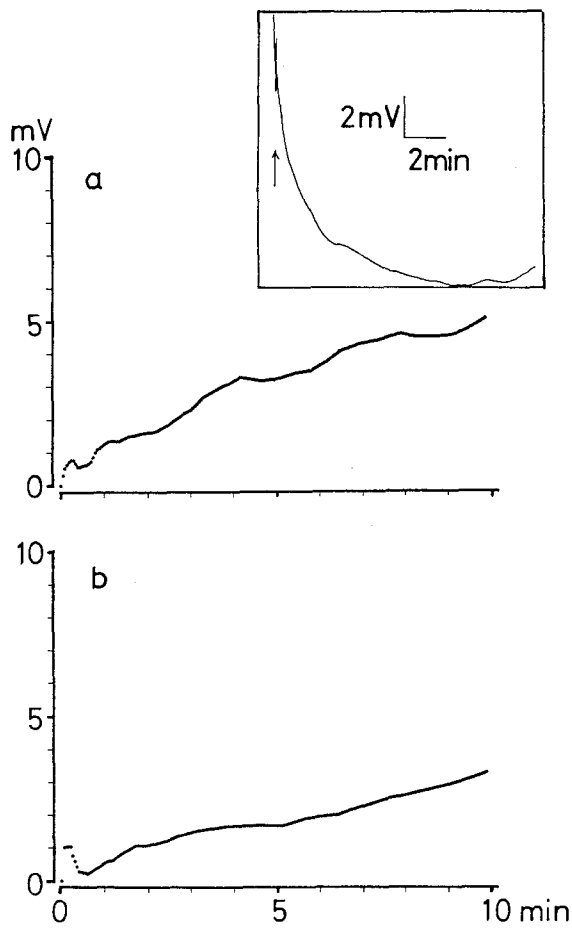

Figure 3. Inhibition of DEP-induced calcium release by magnesium and ruthenium red. *a* The medium contained 5 mM, instead of 1 mM, $MgCl_2$. 0.96 mM DEP. *b* Ruthenium red (final 5 µM) was added to the medium together with 0.91 mM DEP (1 mM $MgCl_2$). Both *a* and *b* are calculated traces as in figure 2. Inset shows the original recording for a. Ca_T: 99 µM (*a*), 98 µM (*b*). Slope of the electrode value per pCa unit: 28.4 mV (*a*), 27.4 mV (*b*). Value at 5 min: 3.3 mV (*a*), 1.7 mV (*b*), Ca_{free} at 5 min: 2.13 µM (*a*), 1.22 µM (*b*). Released calcium at 5 min in nmol/mg SR protein (% of taken-up calcium): 0.8 (1.2%) (*a*), 1.8 (3.3%) (*b*). Value at 10 min: 5.0 mV (*a*), 3.3 mV (*b*). Ca_{free} at 10 min: 1.98 µM (*a*), 1.16 µM (*b*). Released calcium at 10 min in nmol/mg SR protein (% of taken-up calcium): 1.0 (1.6%) (*a*), 3.1 (5.5%) (*b*).

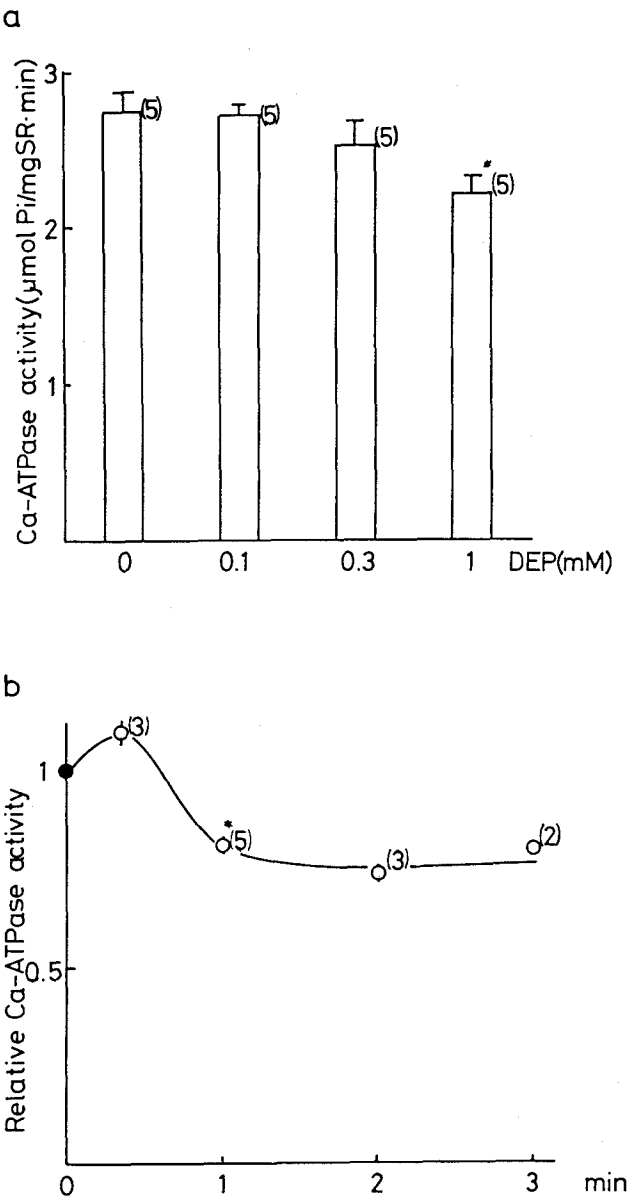

Figure 4. Inhibition of Ca-ATPase activity by DEP. *a* Dose dependency. The enzyme activity was measured following exposure of the SR to DEP for 1 min. *b* Time course of the inhibition of relative activity by 1 mM DEP. Vertical bars and figures in parentheses show SEM and numbers of samples examined, respectively. * indicates $p < 0.05$, by Wilcoxon signed-rank test.

the experimental condition; for example, the main cation, SR concentration, and DEP concentration (not described in Shoshan-Barmatz's paper). However, no further study was attempted here.

The inhibition of Ca-ATPase by DEP was not affected by magnesium. Activity in 1 mM DEP relative to control was 0.97 ± 0.20 (n = 4) or 0.86 ± 0.13 (n = 5) in 1 mM or 3 mM $MgCl_2$, respectively. One mM DEP reduced the enzyme activity to 0.81 ± 0.02 of the control in the presence of 5 μM ruthenium red. These results indicate that inhibition of Ca-ATPase is not the direct cause of calcium release by DEP. It may be that DEP modifies calcium-releasing mechanisms, for example, the calcium channel itself or the receptor for signals from transverse tubules, and enhances the release of calcium from SR.

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Depressor effects of muscarinic and non-muscarinic mediation induced by lateral hypothalamic stimulation in the cat

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Summary. Transient sympathetically-mediated depressor effects were induced by stimulation of a small locus in the lateral hypothalamic peri-fornical region, medial to the fields of Forel. The ganglionic blocking agent, atropine methyl nitrate (ATMN), was used to show that muscarinic as well as non-muscarinic sympathetic ganglion receptor neurotransmission was involved. Evidence is presented that stimulation of this LH site co-activates a number of mechanisms and that depending on which of these are activated, the ganglionic blocking agent ATMN may either block, reverse or potentiate the depressor effect.
Key words. Hypothalamic-induced depressor effects; muscarinic-mediated BP; depressor effect blockage; depressor effect reversal; depressor effect potentiation.

Transient changes in blood pressure (BP) and heart rate (HR) normally take place in response to specific situational stimuli. These in turn activate antagonistic mechanisms that act to re-establish homeostatic conditions. However, disturbed autonomic patterns may persist due to failure of stabilizing mechanisms [7, 16, 17], possibly mechanisms other than the better known baroreceptor system [12].

A study in search of alternative control mechanisms was undertaken on an experimental model developed by us for the study of transient disturbances in BP and HR [3–5]. In this model, stimulation of specific sites in the lateral hypothalamus (LH) induces transient pressor and/or depressor effects, that are uniquely not associated with a change in HR [5].

In a previous study on a pressor effect induced by stimulation of an adjacent site, a sympathetic, nicotinic-receptor ganglionic mediation was revealed [4, 5]. We also found a tendency towards HR constancy or for small HR increases. Along with the BP rise, these were accounted for on the basis of baroreceptor suppression by such stimulation [11]. We have previously reported lack of vagal involvement [3]. Inhibitory mechanisms, presumably of the type described by Libet [14, 15] in sympathetic ganglia, were postulated to be involved in interactions with the above-mentioned neurotransmission mechanisms, to attenuate them. Their blockage was assumed to lead to the potentiation previously reported for the pressor effects, and observed in the present work for the depressor effects. Evidence was obtained for both muscarinic and