13 Kravtsov GM, Kwan CY. A revisitation on the mechanism of action of KCl-induced vascular smooth muscle contraction: a key role of cation binding to the plasma membrane. Biol Signals 1995; 4: 160 - 7.

14 Rasmussen H, Takuwa Y, Park S. Protein kinase C in the regulation of smooth muscle contraction. FASEB J 1987; 1: 177 - 85.

15 Haller H, Smallwood JI, Rasmussen H. Protein kinase C translocation in intact vascular smooth muscle strips. Biochem J 1990; 270: 375 - 81.

## 蛋白激酶 C 不参与在无钙溶液内高钾引起的血管收缩



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关键词 钙; 蛋白激酶 C; Calphostin C; 吩噻醇酯类; 血管平滑肌

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# Suppression of channel conductance by diacetyl monoxime in guinea pig and embryonic chick cardiomyocytes

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**KEY WORDS** oximes; diacetyl monoxime; myocardium; cultured cells; calcium channels; sodium channels; potassium channels; patch-clamp techniques

**AIM**: To examine the effects of diacetyl monoxime (DAM), and putative dephospohrylating agent, on conductances of the cardiac Ca, Na, and K channels. **METHODS**: The Ca ($I_{Ca}$), Na ($I_{Na}$), and K ($I_K$) currents were recorded in single ventricular myocytes from guinea pigs and chick embryos before and after addition of DAM using the whole-cell voltage-clamp technique. **RESULTS**: DAM 10 mmol·L$^{-1}$ reduced rapidly the amplitudes of $I_{Ca}$ (by about 30 %), $I_{Na}$ (by about 25 %), and $I_K$ (by 25 % - 50 %) without alterations of the voltage-dependence. **CONCLUSION**: DAM was a channel inhibitor of the unique type having nonselective phosphatase activities.

Diacetyl monoxime (DAM) was used for the treatment of organophosphorus poisoning, in which acetylcholinesterase is reactivated by the oxime[1]. Because of its nucleophilic properties shared with oximes, DAM was suggested to possess a dephosphorylating (phosphatase-like) action, which is evidenced via an interference with cAMP-dependent phosphorylations from the cytoplasmic side[2,3]. Since the activity of the Ca[4], Na[5], or K channel[6] is controlled by phosphorylations of many types and steps, it is of interest whether DAM deactivates such channels through its phosphatase-like properties.

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## MATERIALS AND METHODS

Voltage-clamp (v - c) experiments were carried out using ventricular myocytes from chick embryos (cultured for 5 - 60 h) and guinea pigs (dispersed 1 - 6 h before each experiment)[7]. Ventricular myocytes from guinea pigs were obtained[8].

Using the whole-cell v - c method, membrane currents were recorded. Pipettes were Sylgard-coated and heat-polished. The composition of the pipette solution was (mmol·L$^{-1}$): K- or Cs-aspartate 130, egtazic acid (EGTA) 11, $CaCl_2$ 1, NaCl 10, $MgCl_2$ 2, $Na_2ATP$ 5, glucose 5, and HEPES 10.0 (pH 7.2). The composition of the bath solution (superfusate) was (mmol·L$^{-1}$): NaCl 140, CsCl or KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, Na-pyruvate 5, glucose 10, and HEPES 10 (pH 7.4). In experiments of the Ca ($I_{Ca}$) and K currents ($I_K$), tetrodotoxin (TTX) 0.01 mmol

$\cdot L^{-1}$ was added to the superfusate. The experiments were carried out at either 26 – 28 ℃ ($I_{Ca}$ and $I_K$ studies) or 17 ℃ ($I_{Na}$ study) controlled by a Peltier-effect unit. The voltage values were corrected for the liquid junction potential of 15 mV.

The cell membrane capacitance was 5 – 10 pF for the embryonic chick myocyte and 70 – 110 pF for the guinea pig myocytes, when measured by ramp-pulses (dV/dt = 0.5 V/s for guinea pig and 5 V/s for embryonic chick myocytes). The series resistance ($R_s$) was 60 % – 70 % compensated before each experiment. Data from experiments in which the residual $R_s$ after its compensation exceeded 10 MΩ were discarded. Pulses were delivered either at 0.1 Hz ($I_{Ca}$ study) or at 0.5 Hz ($I_{Na}$ study). In $I_K$ study, the ramp-pulse protocol (dV/dt = about 20 mV/s) was used. A holding potential ($V_H$) was either – 40 to – 45 mV ($I_{Ca}$ study), – 90 mV ($I_{Na}$ study), or – 70 mV ($I_K$ study). The magnitude of peak $I_{Ca}$ and $I_{Na}$ is defined as the difference between the current peak and the current level at the end of pulses of 200 ms long.

The concentration of DAM was 10 mmol $\cdot L^{-1}$, taking account of DAM concentrations (0.5 – 20 mmol $\cdot L^{-1}$) in earlier works[9-12]. All data were $\bar{x} \pm s$, compared with paired $t$-test.

## RESULTS

### Ca channel conductance

**Current (I) – voltage (V) relation** Using the whole-cell v – c method, the L-type Ca current ($I_{Ca}$) was recorded during exposure of embryonic myocytes ($C_m$ 5 – 10 pF) to DAM 10 mmol $\cdot L^{-1}$ in the presence of TTX 0.01 mmol $\cdot L^{-1}$ (Fig 1A – C). Fig 1A shows v – c records of the Ca currents in an embryonic chick ventricular myocyte, and Fig 1B shows the $I - V$ relation. In the absence of DAM ($n = 14$), the $I_{Ca}$ reached the maximum ($I_{max}$) of – 51 to – 245 (– 102 ± 74) pA at 3.2 ± 10.8 mV ($V_p$).

The most marked change in $I_{Ca}$ after DAM addition was a reduced amplitude of the current (Fig 1A & B). The extent of the DAM-induced reduction of $I_{Ca}$, about – 30 %, was similar at any test voltage (Fig 1C). The effect of DAM on $I_{Ca}$ was readily abolished by washout of myocytes with a drug-free solution, as reported by others[11,12]. In 7 experiments after DAM, the value of $V_p$ after DAM was 3.6 ± 10.0 mV, comparable to that before DAM. A similar suppressing action was seen in ventricular myocytes from guinea pigs

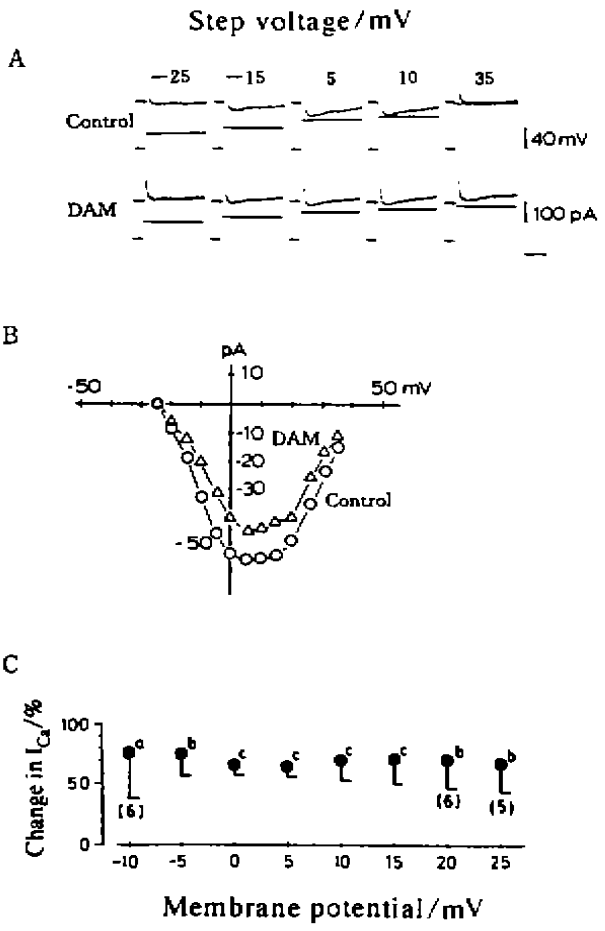



**Fig 1. Effects of DAM on L-type Ca current $I_{Ca(L)}$. A: Response to single pulse steps to various potentials from a holding potential ($V_H$) of – 45 mV in an embryonic chick heart cell. Upper tracing = current; lower tracing = step voltage. Top and bottom rows = before & 3 min after DAM, respectively. B: Peak current (I) – voltage (V) relation after DAM 10 mmol $\cdot L^{-1}$ (Data in A). C: Change in $I_{Ca(L)}$. $n = 7$, unless specified in parentheses. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$.**

(Fig 2). In these cells ($n = 7$), the magnitude of the peak current after DAM relative to that before DAM was 70 % ± 16 % ($P < 0.01$), which was identical to the $I_{Ca}$-reduction in chick embryonic myocyte. Hence, DAM suppressed the Ca conductance.

**Steady-state inactivation ($f_\infty$)** To examine the voltage-dependence of DAM actions on the Ca channels, the steady-state inactivation ($f_\infty$) characteristics was examined. Fig 3A illustrates a

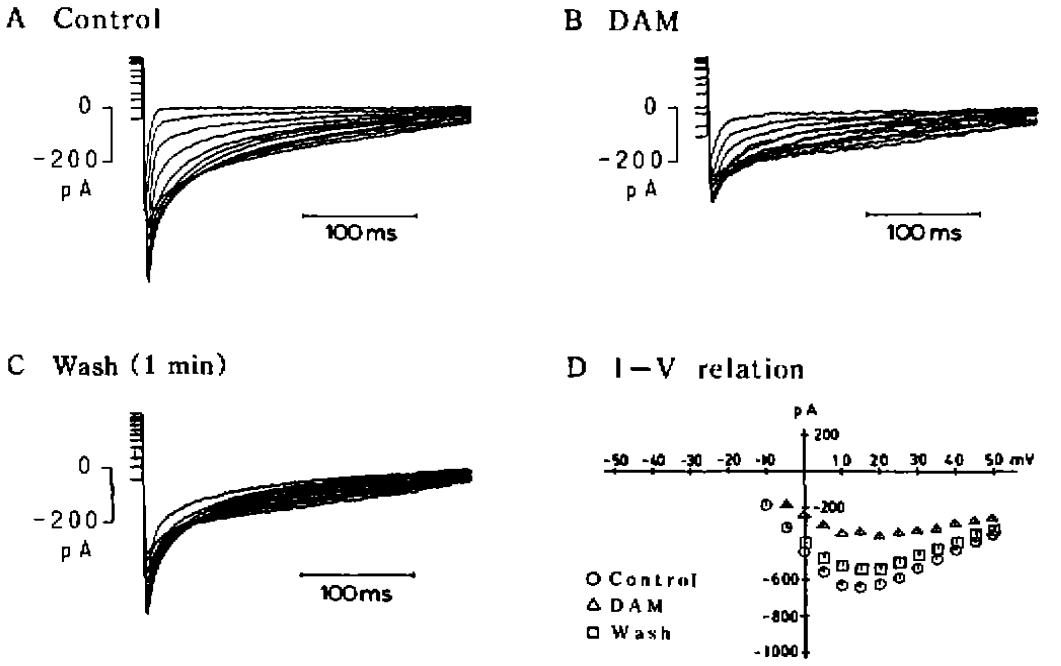



**Fig 2.** $I_{Ca(L)}$ in a guinea pig myocyte. Response to voltage steps of increasing amplitudes in 5 mV steps are superimposed. $V_H = -40$ mV. A: Before DAM. Voltage steps are between $-10$ and $+50$ mV. B: After DAM 10 mmol·L$^{-1}$. Voltage steps are between $-5$ and $+50$ mV. C: 1 min after washout of DAM. Voltage steps are between 0 and $+50$ mV. In A − C, the 0 current denotes the current level at the end of the pulse of 200 ms long. D: I − V relation.

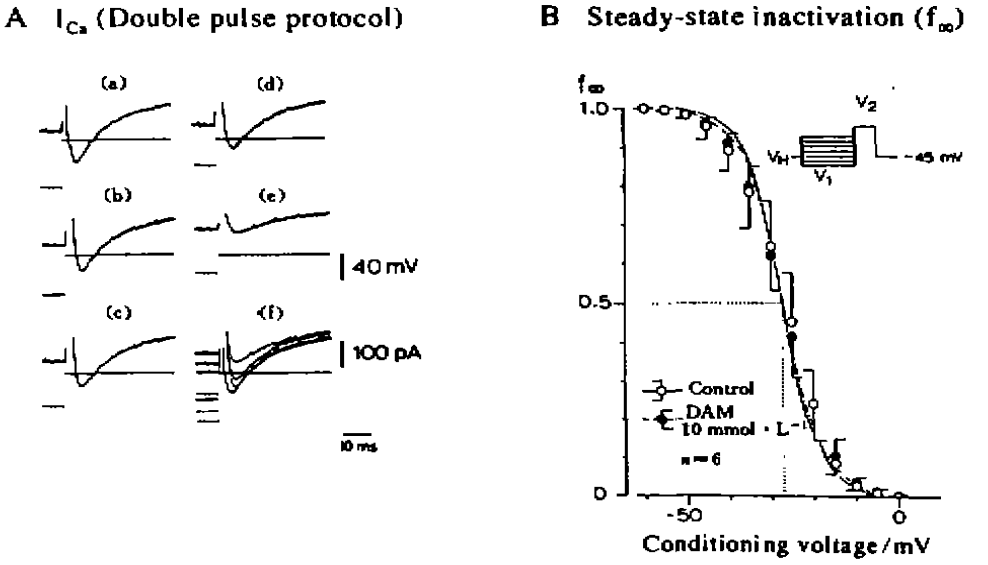



**Fig 3.** Steady-state inactivation of Ca channel. A: Current in a 17-d-old embryonic chick heart cell in the presence of TTX 0.01 mmol·L$^{-1}$. The pulse protocol is shown in inset in B. The test pulses ($V_2$), 200 ms long, to $+12$ mV were preceded by the conditioning pulses ($V_1$), 500 ms long, to $-60$ (a), $-45$ (b), $-40$ (c), $-30$ (d), and $-15$ mV (e). In (f) are superimposed currents in response to $V_2$ pulses preceded by $V_1$ pulses to $-60$, $-45$, $-30$, and $-20$ mV (from top to bottom). B: Summary of 6 experiments for steady-state inactivation study before (○) and after (●) DAM addition. Peak current values ($\bar{x} \pm s$) relative to the maximal current as a function of voltage, in accordance with Boltzmann equation for control (——) and DAM (·····). $V_{half}$ and $S$ were, respectively, $-27.2$ and 4.3 mV for the control and $-27.1$ and 4.8 mV for DAM. The broken line illustrates the $V_{half}$ values.

double-pulse experiment to examine the $f_\infty$. Plots of the magnitude of $I_{Ca}$ in response to the test steps ($V_2$) against the $V_1$-voltage reflected the degree of the steady-state inactivation during the conditioning steps ($V_1$). Fig 3B demonstrates that sigmoidal voltage-dependence of $f_\infty$ before and after DAM addition were superimposable. Values ($\bar{x} \pm s$) of the $V_{half}$ and slope factor ($S$) for $f_\infty$, when fitted tothe Boltzmann equation:

$$f_\infty = 1/\{1 + EXP[(V_m - V_{half})/S]\}$$

were, respectively, $-27.2 \pm 5.7$ and $4.3 \pm 1.4$ mV before, and $-27.1 \pm 4.6$ and $4.8 \pm 1.2$ mV after exposure to DAM. Because DAM did not affect the $f_\infty$ curve, the suppression of the Ca conductance by DAM was concluded to be voltage-independent.

**Na channel conductance** The fast Na current ($I_{Na}$) was recorded in embryonic chick ventricular myocytes ($C_m = 5 - 10$ pF) at 17 ℃ in the external Na concentration of 140 mmol·L$^{-1}$. All K ions in the bath and pipette solutions were replaced with the equimolar Cs ions. At a holding potential of $-90$ mV, currents in response to test steps of 50 ms-long between $-60$ and $+60$ mV in 4 mV increments were recorded. Fig 4 shows representative original $I_{Na}$ records before (A) and after exposure to DAM 10 mmol·L$^{-1}$(B). The current began to flow at ($V_{TH}$) about $-60$ mV, reached the maximum ($V_p$) at about $-15$ mV, and reversed the sign from negative to positive at ($V_{rev}$) about $+55$ mV. Exposure to DAM 10 mmol·L$^{-1}$ (Fig 4B) was immediately followed by a reduction of $I_{Na}$ amplitude. The change in peak $I_{Na}$ is illustrated with the current ($I$)-voltage ($V$) relation (Fig 4C). Neither the rates of activation and inactivation nor the $I - V$ parameters such as $V_{TH}$, $V_p$, and $V_{rev}$ was altered by DAM addition. On average ($n = 4$), DAM reduced the peak amplitude of $I_{Na}$ by $-24 \pm 14$ ($\bar{x} \pm s$, $P < 0.05$), when assessed at $V_p$ (ie, $-15$ mV). Hence, DAM reduced $I_{Na}$ simply by suppressing the limiting conductance of the Na channel ($\overline{G_{Na}}$).

**K channel conductance** The ramp-pulse protocol reveals the inwardly-rectifying K current ($I_{K1}$). the inward rectifier current was recorded in

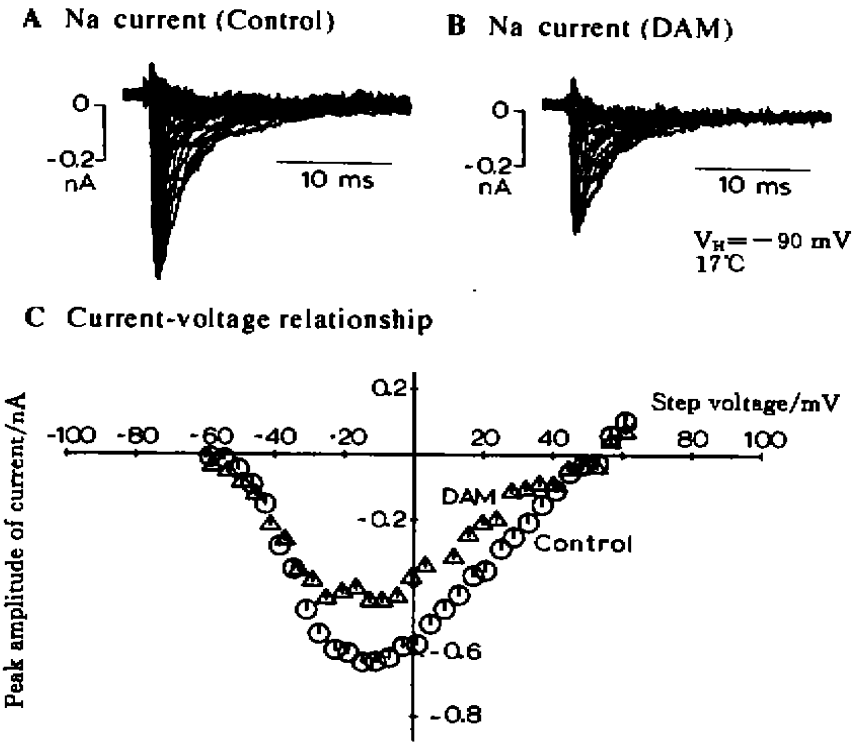
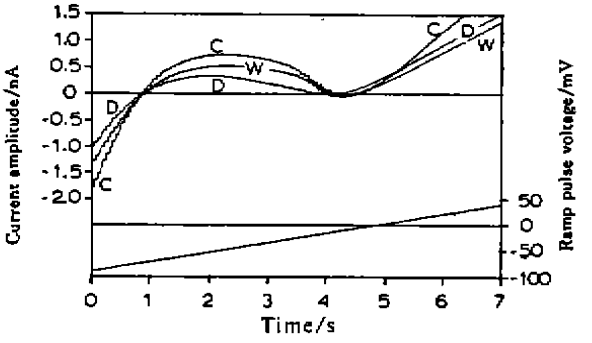



**Fig 4. Fast Na current in a 17-d-old embryonic chick myocyte. A: Before DAM. B: 3 min after DAM 10 mmol·L$^{-1}$. C: Current-voltage relation. In A & B, superimposed are tracings of currents in response to voltage steps of increasing amplitudes from $-60$ mV and $+60$ mV in 4 mV steps at a holding potential of $-90$ mV. $K^+$ in pipette and bath solutions were substituted with $Cs^+$.**

guinea pig ventricular myocytes in the presence of TTX (0.01 mmol·$L^{-1}$) and $Co^{2+}$ ion (0.5 mmol·$L^{-1}$) to block the fast Na and Ca currents, respectively, and its changes by DAM were tested. At a holding potential of −70 mV, ramp-pulses, the slope of which was about 20 mV/s, were given.

Fig 5A illustrates representative inward rectifier currents, and Fig 5B shows the $I-V$ relations. Currents in response to ramp-pulses showed an N-shaped, inwardly-rectifying, property: concomitantly with a progressive depolarization (Fig 5B), the current altered sign from inward to outward at ($V_{rev}$) about −80 mV, then crept over a potential range from $V_{rev}$ to −10 mV exhibiting a negative slope region and increased its amplitude again toward the outward direction, thereafter. As shown in Fig 5A and B, DAM 10 mmol·$L^{-1}$ reversibly suppressed the inward and outward components of the rectifier curent ($I_K$). In total ($n=4$), the extent of the reduction of $I_{K1}$ was (%, *vs* the control levels): −52 ± 22 ($P<0.05$) at −80 mV, −38 ± 14 ($P<0.05$) at −50 mV, and −26 ± 16 ($P<0.05$) at +40 mV. Hence, DAM suppresses the K channel conductance, too.

A Inward rectifier current (ramp-pulse study)




B Current – voltage relation

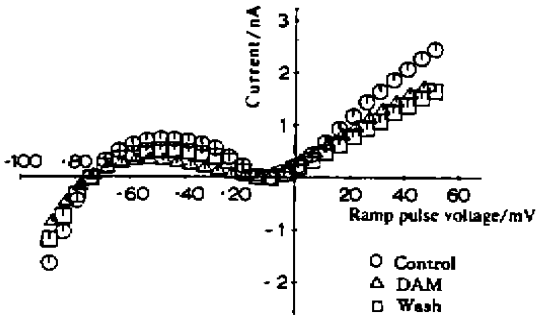



**Fig 5. Inward rectifier current ($I_{K1}$) in a guinea pig myocyte in the presence of TTX (0.01 mmol·$L^{-1}$) and $Co^{2+}$ (0.5 mmol·$L^{-1}$). A: Current tracings (dV/dt = 20 mV/s). Labels C, D, and W denote control period, DAM 100 mmol·$L^{-1}$, and 1 min after washout, respectively. B: Current-voltage relation of result in ramp-pulse study.**

## DISCUSSION

DAM effects in the microelectrode studies with ventricular preparations of cats[9], dogs[10], guinea pigs[11], rabbits[12], and chick embryos[12] were: (1) negative inotropic effects[9,11,13]; (2) a shortening of the duration of the fast and slow action potentials (APS)[9-12]; (3) a suppression of the maximal rate of rise ($V_{max}$) or amplitude of the fast and slow APS[11,12]; (4) small reductions of the resting membrane potential[11,12], and (5) the accelerated automaticity[12]. All these actions suggested reductions of the conductances of channels of any types in cardiac tissues.

We found that, in embryonic chick and guinea pig ventricular myocytes, DAM reduced the conductance of all the channles tested, without affecting the voltage-dependent kinetics. Although Coulombe *et al*[14] reported a lengthening, instead of shortening, of the AP duration in rat ventricular myocytes, they concluded that such an apparent lengthening was derived from a reduction of the conductance of the transient outward current, a K-permeant channel which was aboundant especially in the ventricular myocyte of rat.

DAM has a nonspecific dephosphorylating activity[1-3]. The experimental results herein well elucidates the findings so far obtained in AP-recording studies. Also, our data not only show that the action of DAM on ion channel conductances are always depressant rather than stimulatory, but also reinforce a notion that activation of ion channels of any type require phosphorylation steps, whether the phosphorylations are cAMP-dependent. Although the phosphatase activity of DAM seems to be nonspecific, utilization of DAM will be much helpful in electrophysiological and pharmacological studies.

## REFERENCES


1 Sellin LC, McArdle JJ. Multiple effects of 2, 3-butanedione monoxime. *Pharmacol Toxicol* **1994**; **74**: 305 - 13.

2 Huang G-J, McArdle JJ.
Novel suppression of an L-type calcium channel in neurones of murine dorsal root ganglia by 2,3-butanedione monoxime
J Physiol (Lond) 1992; **447**: 257 - 74.

3 Chapman RA. The effect of oximes on the dihydropyridine sensitive Ca current of isolated guinea-pig ventricular myocytes.
Pflügers Arch 1993; **422**: 325 - 31.

4 Sperelakis N. Regulation of calcium slow channels of hearts by cyclic nucleotides and effects of ischemia.
Adv Pharmacol 1994; **31**: 1 - 24

5 Matsuda JJ, Lee H, Shibata EF. Enhancement of rabbit cardiac sodium channels by β-adrenergic stimulation.
Circ Res 1992; **70**: 199 - 207

6 Olesen SP, Bundgaard M. ATP-dependent closure and reactivation of inward rectifier $K^+$ channels in endothelial cells.
Circ Res 1993; **73**: 492 - 5.

7 Sada H, Kojima M, Sperelakis N. Fast inward current properties of voltage-clamped ventricular cells of embryonic chick heart.
Am J Physiol 1988; **255**: H541 - 53.

8 Mitra R, Morad M. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates.
Am J Physiol 1985; **249**: H1056 - 60.

9 Wiggins JR, Reiser J, Fitzpatrick DF, Bergey JL.
Inotropic actions of diacetyl monoxime in cat ventricular muscle
J Pharmacol Exp Ther 1980; **212**: 217 - 24.

10 Bergey JL, Reiser J, Wiggins JR, Freeman AR.
Oximers: 'enzymatic' slow channel antagonists in canine cardiac Purkinje fibers?
Eur J pharmacol 1981; **71**: 307 - 19.

11 Li T, Sperelakis N, Teneick RE, Solaro RJ Effects of diacetyl monoxime on cardiac excitation-contraction coupling.
J Pharmacol Exp Ther 1985; **232**: 688 - 95

12 Sada H, Sada S, Sperelakis N. Effects of diacetyl monoxime (DAM) on slow and fast action potentials of young and old embryonic chick hearts and rabbit hearts.
Eur J Pharmacol 1985; **112**: 145 - 52.

13 Morner SEJN, Wohlfart B. The action of 2, 3-butanedione-monoxime on the inotropic state in guinea-pig myocardium.
Acta Physiol Scand 1991; **142**: 211 - 9

14 Coulombe A, Lefevre IA, Deroubaix E, Thuringer D, Coraboeuf E. Effect of 2, 3-butanedione 2-monoxime on slow inward and transient outward currents in rat ventricular myocytes.
J Mol Cell Cardiol 1990; **22**: 921 - 32.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# 二乙酰单肟抑制豚鼠和鸡胚心肌细胞离子通道电导

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**关键词** 肟类; 二乙酰单肟; 心肌; 培养的细胞; 钙通道; 钠通道; 钾通道; 膜片箝技术