3) and its block in dorsal root ganglion neurons (6, 7)], the inability to suppress the enhancing effect of the peptide in cardiac cells dialyzed with GDP-β-S or in the presence of α_1- or β-blockers suggests that its effect is not mediated through a G protein–dependent pathway. We do not yet know whether a similar molecular mechanism mediates both the agonistic and antagonistic effects of the peptide on cardiac and neuronal Ca^{2+} channels. Our results, nevertheless, suggest the existence of endogenous peptide in the brain that may serve as a physiological regulator of Ca^{2+} channels by a novel mechanism.

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Specific Block of Calcium Channel Expression by a Fragment of Dihydropyridine Receptor cDNA

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Although the structure of rabbit skeletal muscle dihydropyridine (DHP) receptor, deduced from cDNA sequence, indicates that this protein is the channel-forming subunit of voltage-dependent calcium channel (VDCC), no functional proof for this prediction has been presented. Two DNA oligonucleotides complementary to DHP-receptor RNA sequences coding for putative membrane-spanning regions of the DHP receptor specifically suppress the expression of the DHP-sensitive VDCC from rabbit and rat heart in *Xenopus* oocytes. However, these oligonucleotides do not suppress the expression of the DHP-insensitive VDCC and of voltage-dependent sodium and potassium channels. Thus, the gene for DHP receptor of rabbit skeletal muscle is closely related, or identical to, a gene expressed in heart that encodes a component of the DHP-sensitive VDCC. The DHP-sensitive and DHP-insensitive VDCCs are distinct molecular entities.

SEVERAL TYPES OF VDCCs EXIST; THE most thoroughly studied channel is sensitive to DHPs (L-type) (1). The DHP receptor (DHPR) of skeletal muscle is one of the four or five subunits that presumably constitute the channel (2). Skeletal muscle DHPR may be the voltage sensor of excitation-contraction coupling (3), or be associated with functional VDCCs (4), or may serve both functions (5). The primary structure of the rabbit skeletal muscle DHPR, deduced from cDNA that encodes this protein, is as expected for an ion channel (5). However, the functional expression of the cDNA clones has not yet been demonstrated. To examine whether DHPR is indeed an important component of the channel, we used hybrid arrest of mRNA expression in *Xenopus* oocytes by complementary oligonucleotides (6). With skeletal muscle RNA, we could not achieve reproducible expression of the DHP-sensitive VDCC in the oocytes (7); therefore, we used heart RNA for this purpose.

Frogs were maintained and dissected and the follicles removed from the oocytes, as described (8, 9). RNA was isolated from hearts of 7-day-old rats or 10-day-old rabbits by a LiCl-urea method (10). Total RNA was used in this study; it induced Ca^{2+} channel currents similar to those induced by polyadenylated RNA (9). Injected oocytes were incubated for 3 to 5 days at 22°C in sterile ND96 solution (9) (96 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 5 m*M* Hepes-NaOH; *p*H, 7.5) supplemented with antibiotics and sodium pyruvate before the recording of the currents. Currents through VDCCs were recorded with the two-electrode voltage-clamp technique (9, 11) in a solution containing 40 m*M* Ba^{2+}, 2 m*M* K^+, 50 m*M* Na^+, and 5 m*M* Hepes-NaOH (*p*H, 7.5), the anion being either acetate or methanesulfonate.

Rat and rabbit heart RNA cause the appearance of two distinct VDCC currents in *Xenopus* oocytes (9) (Fig. 1): a transient current (I_{tr}) and a "slow" current (L-type; I_{sl}). I_{sl} was inhibited by DHP blockers (9) and potentiated by the DHP agonist BAY K 8644 (12) (Fig. 1C). I_{tr} was DHP-insensitive and inactivated at more negative voltages than I_{sl} (9) (Fig. 1B). After a complete block of Ba^{2+} currents by 0.5 m*M* Cd^{2+} or by the oligonucleotide treatment, depolarizations to potentials beyond −20 mV evoked only a slow, noninactivating outward current (I_{out}), which rarely exceeded 5 nA at 0 mV at the end of the depolarizing pulse.

The currents were quantified as shown in Fig. 1. I_{sl} was slightly underestimated because of its mild inactivation and the development of I_{out}. Presence or absence of I_{sl} was verified by testing the effect of BAY K 8644 on the currents (Table 1). The amplitude of I_{tr} was estimated by two independent procedures. The leak-subtraction procedure (Fig. 1A) overestimates I_{tr} because the total current at the end of the depolarizing pulse contains an outward current component (I_{out}). The "I_{tr}-inactivation" procedure (Fig. 1B) offsets the contribution of the leak current, I_{sl} and I_{out}, but I_{tr} is underestimated by 25 to 30% because its inactivation is incomplete at −20 mV (9).

Although the transient, BAY K 8644–insensitive Ba^{2+} current in native (not injected with RNA) oocytes (9, 10, 13) (Fig. 1C, part a) resembles the I_{tr} in heart RNA–injected oocytes, I_{tr} is always at least two (usually three to five) times larger than the native current (Table 1) (7). The possibility that the native VDCC is overexpressed in heart RNA–injected cells is unlikely for several reasons (14). The primary reason is that the expression of endogenous messages is reduced by exogenous RNA (15). Thus, we postulate that a major part of I_{tr} is directed by heart RNA.

The method for selective inhibition of RNA expression in *Xenopus* oocytes is based on work (16) that demonstrates that DNA

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oligonucleotides (15 to 30 nucleotides long) complementary to an mRNA sequence specifically inactivate this mRNA in *Xenopus* oocytes by a RNase H–like degradation mechanism. (RNase H is an enzyme that hydrolyzes the RNA moiety of RNA-DNA hybrids.) This method has been modified (*17*) to include prolonged hybridization of heterologous RNA with the oligonucleotides before the injection into the oocytes. The early hybridization improves the efficiency of the expression arrest caused by the oligonucleotide and enables selective suppression of rare messages coding for channel proteins (the percentage of which in heterologous RNA is low). The hybridization procedure included denaturing RNA by brief heating (65°C for 2 min) and its incubation without or with one of the oligonucleotides in 200 m*M* NaCl and 10 m*M* tris-HCl (*p*H 7.4) for 3 to 4 hours at 37°C before injection into oocytes (50 nl per oocyte). Final concentrations of rat and rabbit heart RNAs were 4.2 mg/ml.

We used two oligonucleotides to arrest the VDCC expression. DNA oligonucleotides were synthesized by phosphoramidite chemistry with a DNA synthesizer (Applied Biosystems). cDHPR-O_1 was 57 nucleotides long, complementary to the rabbit skeletal muscle DHPR mRNA (*5*) (sequence position 3693 to 3750). This stretch putatively codes for an "S4" segment implicated as the voltage sensor of the presumptive Ca channel. cDHPR-O_2 was 80 nucleotides long and complementary to position 2560 to 2640 in the above sequence (the presumptive transmembrane segment S3 plus an extramembrane stretch). We used these sequences because membrane-span-

Table 1. The effect of cDHPR-O^1 on the expression of heart Ca^{2+} channel in oocytes: summary of experiment 1. The experiment was performed with oocytes of one frog. The entries are mean ± SD; number of cells tested is shown in parentheses. ND, not determined.

RNA and treatment	Currents (nA)			BAY K 8644–induced current changes (nA)†		
	Total‡	I_{sl}‡	I_{tr}§	Total‡	I_{sl}‡	I_{tr}§
No RNA	−9 ± 3 (5)	+1 ± 2 (5)	−5 ± 1 (4)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)
Rabbit	−41 ± 10 (12)	−17 ± 9 (12)	−10 ± 3 (9)	−43 ± 19‖ (8)	−50 ± 41‖ (8)	0 ± 1 (6)
Rabbit cDHPR-O_1 (2.5 μg/ml)	−25 ± 11* (5)	−1 ± 12* (5)	−15 ± 3 (3)	−12 ± 8‖ (5)	−15 ± 8‖ (5)	+2 ± 2 (3)
Rabbit cDHPR-O_1 (25 μg/ml)	−20 ± 8** (11)	+5 ± 9** (11)	−14 ± 6 (5)	−1 ± 4 (7)	−1 ± 3 (7)	0 ± 1 (5)
Rat	−75 ± 20 (7)	−37 ± 16 (7)	−23 ± 11 (3)	−54 ± 28‖ (4)	−71 ± 36‖ (4)	−4 ± 2 (2)
Rat cDHPR-O_1 (25 μg/ml)	−74 ± 30 (3)	−29 ± 16 (3)	−20 ± 9 (3)	−38 ± 15‖ (3)	−59 ± 35‖ (3)	+6 (1)
Rat cDHPR-O_1 (25 μg/ml)	−44 ± 17*** (6)	−5 ± 6** (6)	ND	−12 ± 5‖ (3)	−18 ± 9‖ (3)	ND

*$P < 0.025$. **$P < 0.01$. ***$P < 0.05$. The significance of the reduction of the curent by cDHPR-O_1 treatment was tested by one-tailed *t* test, adjusted by a Bonferroni-type correction for multiple comparisons. †The change in current caused by 1 μ*M* BAY K 8644 was either negative (increase in the inward current) or positive (decrease in the inward current). It was calculated in each cell treated with BAY K 8644. ‡Calculated by the leak-subtraction procedure. §Calculated by the I_{tr}-inactivation procedure. ‖Indicates a significant ($P < 0.05$) increase in inward current.

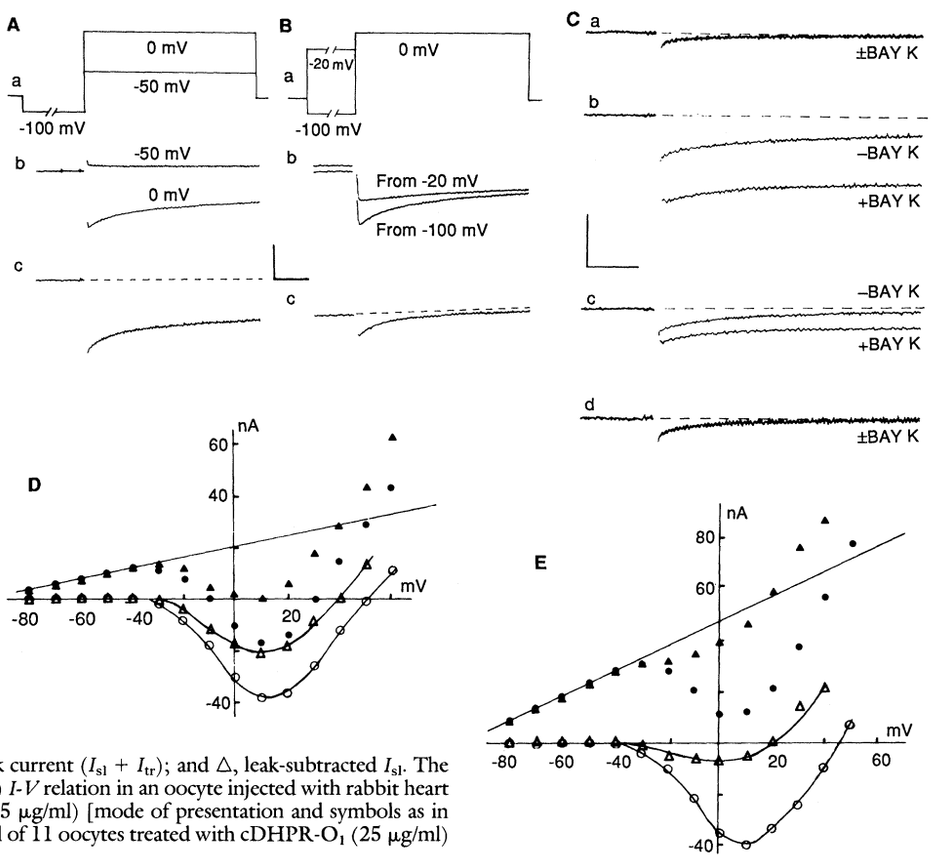

Fig. 1. The effect of cDHPR-O_1 on Ba^{2+} currents in oocytes injected with heart RNA. All currents were recorded in oocytes of one frog. (**A**) The leak-subtraction procedure. (a) The voltage sequence (intervals between steps were 15 s). The duration of the conditioning prepulse was 5 s. (b) The currents recorded in an oocyte injected with rat heart RNA (4.2 mg/ml). (c) The net active current at 0 mV, obtained by scaling and subtracting the current recorded at −50 mV. The current at the end of the pulse, when I_{tr} completely fades, was taken as the estimate of I_{sl}. The net I_{tr} was calculated from this trace by subtracting the current at the end of the pulse from the peak current. (**B**) The I_{tr}-inactivation procedure. (a) The voltage sequence. (b) Currents recorded in a rat heart RNA–injected oocyte. (c) The net Ba^{2+} current inactivated by the prepulse to −20 mV. Obtained by subtraction of the currents evoked by depolarizing steps from −100 and from −20 to 0 mV. The residual inward current at the end of the 2.5-s pulse reflects the inactivation of I_{sl}; it was therefore subtracted from the peak current to obtain the net I_{tr}. (**C**) The effect of BAY K 8644 (1 μ*M*) on Ba^{2+} currents in a native (not injected with RNA) oocyte (a), and in oocytes injected with rabbit heart RNA alone (b), hybridized to cDHPR-O_1 (2.5 μg/ml) (c), or cDHPR-O_1 (25 μg/ml) (d). The currents were obtained by the leak-subtraction procedure. (**D**) *I-V* relation in an oocyte injected with rabbit heart RNA. Symbols are as follows: ●, total peak current; and ▲, current 2.5 s after the peak; ○, leak-subtracted peak current ($I_{sl} + I_{tr}$); and △, leak-subtracted I_{sl}. The straight line shows the extrapolated leak current. (**E**) *I-V* relation in an oocyte injected with rabbit heart RNA that had been incubated with cDHPR-O_1 (25 μg/ml) [mode of presentation and symbols as in (D)]. This is one of the two oocytes [out of the total of 11 oocytes treated with cDHPR-O_1 (25 μg/ml) in experiment 1] that still displayed a residual I_{sl}.

ning stretches of voltage-sensitive channels (especially S4) are most strongly conserved among different species and tissues (*18*).

Two experiments on the effects of cDHPR-O_1 on Ba^{2+} currents were performed. Experiment 1 is presented in Table 1 and Fig. 1. In this experiment, a low concentration of cDHPR-O_1 (2.5 μg/ml) considerably reduced, and 25 μg/ml almost completely eliminated, the BAY K 8644–sensitive I_{sl} in rabbit heart RNA–injected oocytes, whereas I_{tr} was unaltered (Fig. 1C and Table 1) (*19*). The effect on rat heart RNA–directed currents was similar but less pronounced (Table 1). Similar results were obtained in experiment 2 with cDHPR-O_1 at 25 μg/ml: rabbit heart RNA–directed I_{sl} was fully suppressed ($P < 0.005$), and rat heart RNA–directed I_{sl} was reduced by 88% ($P < 0.01$). In both cases, there was no significant change in I_{tr}.

The residual I_{sl} in cDHPR-O_1–treated oocytes retained full sensitivity to BAY K 8644 (Fig. 1C and Table 1). The cDHPR-O_1 treatment did not alter the general shape of the current-voltage (*I-V*) relation of I_{tr} and I_{sl} (Fig. 1, D and E). These results indicate that cDHPR-O_1 decreased the amount of the active message of the DHP-sensitive VDCC, rather than modified its properties. The inactivation properties of I_{Ba} also did not exhibit obvious changes. cDHPR-O_2 was tested in rabbit heart RNA–injected oocytes. Its effects were similar to those of cDHPR-O_1 (two experiments) (Table 2).

To verify the specificity of the above effects, we performed several control experiments. Neither cDHPR-O_1 or cDHPR-O_2 suppressed the expression of the voltage-dependent, tetrodotoxin-sensitive Na^+ current (I_{Na}) or the voltage-dependent transient K^+ current (I_A) in the oocytes injected with chick or rat brain RNA (Table 3) (*20*). Moreover, hybridization of the rabbit heart RNA with two control oligonucleotides, complementary to mRNA sequences coding for presumptive membrane and extracellular stretches of the rat brain voltage-dependent Na^+ channel (*18*), did not reduce the I_{Ba} (Table 3). These oligonucleotides did inhibit the expression of the Na^+ channel in oocytes (*17, 21*). These results demonstrate the specificity of cDHPR-O_1 and cDHPR-O_2 toward the DHP-sensitive Ca^{2+} channel but not toward other voltage-dependent channels. They also prove that the inhibition of the expression of this channel was not due to its lability to any oligonucleotide treatment.

Our results demonstrate that the cDNA of skeletal muscle DHPR cloned by Tanabe *et al.* (*5*) codes for a protein that is highly homologous to a key component of heart Ca^{2+} channel of the L-type. It is probable that this protein has the same function in the skeletal muscle. Yet, the same sequence, associated with different subunits, may play a different role in muscle, or serve a dual function, both as voltage sensor of excitation-contraction coupling and as a Ca^{2+} channel (*3, 5*). Our data also suggest that the DHP-insensitive VDCC is a molecular entity separate from the DHP-sensitive VDCC, although they do not exclude the possibility that the two channels are structurally related or share common subunits.

Note added in proof: Tanabe *et al.* (*22*) reported the restoration of slow Ca^{2+} channel function in dysgenic mice muscle by expressing the DHP receptor from the cloned message, supporting the notion that this clone indeed codes for an essential component of the skeletal muscle Ca^{2+} channel.

Table 2. The effect of cDHPR-O_2 on the expression of rabbit heart Ca^{2+} channels. Each experiment was performed with oocytes of one frog. The entries are currents in nanoamperes (mean ± SD); number of cells tested is shown in parentheses. Negative values correspond to inward currents, positive values correspond to outward currents (which are apparent in cells lacking I_{Ba}). I_{tr} was estimated by the leak-subtraction procedure.

Treatment	Experiment 3		Experiment 4	
	I_{sl}	I_{tr}	I_{sl}	I_{tr}
No cDHPR-O_2	−17 ± 3 (4)	−13 ± 1 (4)	−10 ± 6 (6)	−15 ± 7 (6)
cDHPR-O_2 (8 to 12 μg/ml)	0 ± 1* (5)	−13 ± 1 (5)	+4 ± 4* (7)	−12 ± 5 (7)

*Inward current significantly ($P < 0.01$) smaller than in oocytes not treated with cDHPR-O_2.

Table 3. Specificity of the effects of the oligonucleotides used in the study. Entries show the mean currents in oocytes treated with the oligonucleotides as percentage of mean currents in oocytes not treated with the oligonucleotides. The number of experiments is shown in parentheses. Each experiment was done with oocytes of one frog; three to eight oocytes were tested for each treatment. Calcium channel currents were measured by the leak-subtraction procedure.

Oligonucleotide	RNA*	Currents			
		I_{Na}†	I_A†	I_{Ba}	
				I_{sl}	I_{tr}
cDHPR-O_1 (25 μg/ml)	Chick brain	135 (2)	178 (2)‡		
cDHPR-O_1 (25 μg/ml)	Rat brain	93 (1)	112 (1)		
cDHPR-O_2 (17 or 67 μg/ml)	Rat brain	83 (2)	105 (1)		
cNa-O_1§ (25 μg/ml)	Rabbit heart			102 (1)	112 (1)
cNa-O_4§ (65 μg/ml)	Rabbit heart			270 (1)‡	140 (1)

*RNA concentrations were chick brain, 5.8 mg/ml; rat brain, 6.6 mg/ml; and rabbit heart, 4.2 mg/ml. †I_{Na} and I_A were recorded with the two-electrode voltage clamp technique, in normal ND96 solution, with the addition of 2 to 3 m*M* 9-anthracenecarboxylic acid to suppress the Ca^{2+}-dependent Cl^- current (*7*), and 30 m*M* tetraethylammonium, which suppressed the delayed rectifier K^+ current (*17*). Net I_{Na} was obtained by recording currents evoked by voltage steps from −100 to −10 mV and subtraction of currents evoked by voltage steps from −20 to −10 mV. Net I_A was obtained by recording currents evoked by voltage steps from −100 to +40 mV and subtraction of currents evoked by voltage steps from −50 to +40 mV. I_{Na} ranged between 200 and 1000 nA, I_A from 50 to 300 nA. ‡Significantly greater than in untreated oocytes. §Oligonucleotides complementary to rat brain Na^+ channel mRNA. cNa-O_1 is 42 nucleotides long targeted to an extracellular portion of the channel (nucleotides 5112 to 5154); cNa-O_4 is 84 nucleotides long targeted partly to a membrane spanning "S6" segment (nucleotides 1215 to 1299). The degree of sequence homology between the different oligonucleotides is cDHPR-O_1/cNa-O_1, 45%; cDHPR-O_1/cNa-O_4, 37%; cDHPR-O_2/cNa-O_1, 45%; and cDHPR-O_2/cNa-O_4, 39%.

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19. Only estimates of I_{tr} obtained by the I_{tr}-inactivation procedure are shown in Table 1. I_{tr} estimated by the leak-subtraction procedure was also unaffected by the oligonucleotide treatment.
20. We did not detect expression of voltage-dependent Na^+ or transient K^+ channels in oocytes injected with heart RNA that induced Ca^{2+} currents.
21. In fact, oligonucleotides complementary to mRNAs of some channels sometimes enhanced the expression of other channels (Table 3) (I. Lotan *et al.*, unpublished data), possibly due to weakening of the competition among different RNA species on the translational machinery of the oocytes (*15*); destruction of one species would be expected to potentiate the expression of others.
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Muscarinic Modulation of Cardiac Rate at Low Acetylcholine Concentrations

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Slowing of cardiac pacemaking induced by cholinergic input is thought to arise from the opening of potassium channels caused by muscarinic receptor stimulation. In mammalian sinoatrial node cells, however, muscarinic stimulation also inhibits the hyperpolarization-activated current (I_f), which is involved in the generation of pacemaker activity and its acceleration by catecholamines. Acetylcholine at nanomolar concentrations inhibits I_f and slows spontaneous rate, whereas 20 times higher concentrations are required to activate the acetylcholine-dependent potassium current ($I_{K,ACh}$). Thus, modulation of I_f, rather than $I_{K,ACh}$, is the mechanism underlying the muscarinic control of cardiac pacing at low (nanomolar) acetylcholine concentrations.

SINUS NODE AUTOMATICITY IS NORMALLY modulated by vagal tone. The action of acetylcholine on K^+ conductance was identified as early as 1958 (*1*) and interpreted at that time as the main basis for the slowing of cardiac pacemaking by the vagus. However, later experiments raised questions concerning the significance of this mechanism in mediating cardiac rhythm under conditions of modest muscarinic receptor activation. In particular, it was observed that during short duration vagal stimulation or exposure to nanomolar concentrations of muscarine, a slowing of spontaneous heart rate occurred without any membrane hyperpolarization (*2*). In addition, no increase in K^+ flux was detected under these conditions (*3*). These data suggest that other mechanisms may be involved in the muscarinic control of cardiac rate.

Acetylcholine (ACh) reduces the slow inward Ca^{2+} current (*4*), and this has been suggested to contribute to the observed effects of ACh on cardiac rhythm (*2, 5*). However, in sinoatrial (SA) node cells, the "pacemaker" current I_f also is strongly depressed by ACh (*6*). ACh acts via inhibition of adenylate cyclase and a decreased production of adenosine 3′,5′-monophosphate (cAMP) to shift the I_f activation curve to more negative potentials (*7, 8*). Thus, the possibility arises that I_f inhibition has a role in the vagal modulation of normal cardiac rhythm. To investigate this, we have compared the action of ACh on I_f and $I_{K,ACh}$ in isolated SA node myocytes.

Rabbit SA node myocytes were isolated by treatment with collagenase and elastase and whole-cell voltage or current clamped (*9*). We used freshly isolated cells plated on petri dishes and superfused with a Tyrode solution containing 140 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 20 m*M* *d*-glucose, and 5.0 m*M* Hepes-NaOH, *p*H 7.4. We added $BaCl_2$ (1 m*M*) and $MnCl_2$ (2 m*M*) to better distinguish I_f changes during voltage clamp steps, when indicated. The temperature in the bath was 35° to 36°C. The internal dialyzing solution contained 10 m*M* NaCl, 130 m*M* potassium aspartate, 2.0 m*M* Mg–adenosine triphosphate (ATP), 0.1 m*M* guanosine triphosphate (GTP), 1.0 m*M* EGTA, and 10 m*M* Hepes-KOH, *p*H 7.2. Test solutions were delivered by a superfusion device consisting of a wide-tipped pipette that could be positioned near the cell under study and that allowed fast (2 to 3 s) solution changes.

Superfusion of myocytes with ACh from 0.003 to 30 μ*M* had differential effects on the currents I_f and $I_{K,ACh}$ that changed with concentration. I_f was activated by hyperpolarizing steps from a holding potential of −35 mV (Fig. 1). Addition of 0.01 μ*M* ACh resulted in a reduction of I_f at −85 mV, consistent with a shift of the I_f activation curve to more negative voltages (*6, 7*). The size of the current I_f was reduced more by 0.1 μ*M* ACh (middle) and was only slightly affected by further increasing the ACh concentration to 1 μ*M* (lower). On the other hand, an increase in K^+ permeability, as detected in changes in the holding current and in the instantaneous current at the onset of voltage steps, could only be observed at 0.1 μ*M* or higher ACh concentrations. In all of the seven cells studied by this protocol, I_f inhibition by ACh occurred at concentrations at least one order of magnitude below that at which $I_{K,ACh}$ activation was observed.

Precise quantitation of this apparent difference in sensitivity of I_f and $I_{K,ACh}$ could

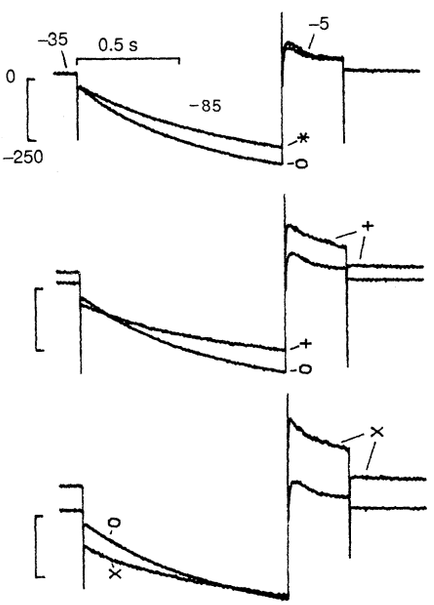

Fig. 1. Separation of the effects of ACh on I_f and $I_{K,ACh}$. Two-pulse protocols were applied every 3 s during superfusion with various doses of ACh. The myocyte was superfused with normal Tyrode solution (○, control) and with Tyrode containing 0.01 (*), 0.1 (+), and 1 μ*M* (x) ACh. In each case ACh superfusion was maintained until a steady-state effect was achieved, typically 20 s, and was followed by an appropriate washout period.

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