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Serum contains a potent factor that decreases β-adrenergic receptor-stimulated L-type Ca^{2+} current in cardiac myocytes

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Abstract. L-type Ca^{2+} current (I_{Ca}) was measured in cultured atrial myocytes from hearts of adult guinea-pigs using whole-cell voltage clamp. Potentiation of I_{Ca} induced by β-adrenergic stimulation (isoprenaline $2 \cdot 10^{-7}$ M) could be completely antagonized by diluted sera (1:100 v/v). Half-maximal inhibition of β-receptor-stimulated I_{Ca} occurred at about 1:1000. Basal I_{Ca} was not affected by serum. Atropine in a concentration (10^{-6} M) that completely antagonized the anti-adrenergic effect of acetylcholine (ACh, $2 \cdot 10^{-6}$ M) did not interfere with the effect of serum. In cells dialysed with cyclic adenosine monophosphate (cAMP)-containing (10^{-4} M) pipette solution, potentiated I_{Ca} was insensitive to both ACh and serum. Preincubation of the myocytes with pertussis toxin almost completely abolished the anti-adrenergic effects of both ACh and serum. The potency of serum was not reduced by dialysis. It is concluded that serum contains a factor which, like ACh, inhibits β-receptor-stimulated adenylyl cyclase via G_i-protein.

Key words: Cardiac myocyte – β-Receptor – Muscarinic receptor – cAMP – G-protein – Serum

Introduction

Cardiac L-type Ca^{2+} current (I_{Ca}) is modulated by phosphorylation catalysed by cyclic adenosine monophosphate (cAMP)-dependent protein kinase (for reviews see [8, 17, 23]). The concentration of cytosolic cAMP is under the control of a number of agonist receptors that are coupled to adenylyl cyclase (AC) via two different types of guanosine nucleotide-binding proteins (G-protein). The classic dual regulation proceeds via β-adrenergic receptors coupled to G_s, resulting in a stimulation of AC, and muscarinic (M2) receptors coupled to G_i, resulting in an inhibition of the G_s-stimulated activity of the enzyme. A number of receptors for other ligands have been shown to be coupled to G-proteins, such as histamine [10] and glucagon [15] to G_s and adenosine [12, 25] and atrial natriuretic peptide [6] to G_i.

We have shown recently that sera from different species contain a factor, not yet identified, that activates a type of inward-rectifying K^+ current ($I_{K,ACh}$) present in supraventricular cells of mammalian heart [1]. $I_{K,ACh}$ is normally controlled by the parasympathetic transmitter acetylcholine (ACh) via muscarinic receptors (see [8, 19] for review). Receptor-mediated $I_{K,ACh}$ activation also proceeds via a G-protein (G_K, probably G_{i2} or G_{i3}). Both $I_{K,ACh}$ activation and AC inhibition by muscarinic agonists can be inhibited by treatment of the cell with pertussis toxin (PTx) [9, 13, 18] which inactivates a certain class of G-proteins by adenosine diphosphate (ADP)-ribosylation of the α-subunit. $I_{K,ACh}$ activation by serum does not involve any of the receptors known to be coupled to these channels, it is nevertheless abolished by pre-treatment of the myocytes with PTx. In the present study it is shown that the unknown serum factor also potently decreases β-receptor-stimulated I_{Ca} via a PTx-sensitive pathway.

Materials and methods

Isolation and culture of atrial myocytes. The method of cell isolation and the conditions for long-term culture of atrial myocytes from hearts of adult guinea-pigs have been described in detail elsewhere [3]. For the experiments cultured myocytes were used from day 0 (freshly isolated) to day 12 after isolation. The membrane capacitance of the cells ranged from 15 to 25 pF.

Solutions. For the measurements the culture medium was replaced by a solution containing (mM): NaCl 140, CsCl 2.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)/CsOH 10.0; pH was 7.4. The solution for filling the patch-clamp pipettes and, thus, internal dialysis of the cells con-

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tained (mM) Cs_3 citrate 60, CsCl 10, NaCl 10, $MgCl_2$ 1.0, HEPES/CsOH 10; pH was 7.4. This solution was supplemented with Mg-adenosine triphosphate (5 mM) and 1 mM [ethylene bis(oxonitrilo)]tetraacetic acid (EGTA). As has been shown previously, inactivation of L-type I_{Ca} is considerably slowed under these conditions [2]. Furthermore, the background *I/V* relation of the cells is very flat and linear in the hyperpolarizing voltage range between −50 and −120 mV [2].

Current measurement. Membrane currents were measured under voltage clamp by means of patch-clamp pipettes (whole-cell mode, [7]). Pipettes were fabricated from Pyrex glass (OD 1.5 mm, ID 1.0 mm) and were filled with the solution above. The DC resistance of the filled pipettes ranged from 2 to 6 MΩ. Current measurements were performed by means of a patch-clamp amplifier (List LM/EPC 7, Darmstadt, Germany). Holding potential was −50 mV throughout, in order to inactivate voltage-dependent I_{Na}. As has been shown previously [2], I_{Ca} in guinea-pig atrial myocytes can be measured without contamination by I_{Na} under the present conditions with this holding potential. Unless otherwise stated, I_{Ca} was elicited by step depolarizations to + 5 mV. For leak subtraction, currents obtained by hyperpolarizing voltage pulses of identical amplitudes as those for activation of I_{Ca} were added to the latter. Signals were stored digitally on the hard disk of an IBM compatible AT computer equipped with an AD/DA board (DT 2821, Data Translation) for subsequent analysis. Experiments were performed at ambient temperature (21−23° C).

Rapid superfusion of the cells for application and withdrawal of different solutions was performed by means of a solenoid-operated flow system that permitted switching between five different solutions. The solutions flowed through capillary tubes into a common outlet. The cell under study was continuously superfused at approximately 0.25 ml/min with a solution stream of 200 µm diameter close to the outlet. The half time for a concentration change in the cell was tested using solutions containing different K^+ concentrations and found to be 200−600 ms, depending on the individual geometrical conditions.

Results

Consistent with previous investigations (reviewed in [8, 17], superfusion of a cardiac cell with an isoprenaline-containing solution resulted in a reversible increase of L-type I_{Ca} evoked by depolarizing voltage steps. In Fig. 1 peak I_{Ca} elicited by step depolarizations to + 5 mV (50 ms duration, 0.15 s^{-1}, holding potential −50 mV) is shown as a function of time. In this cell isoprenaline in a concentration close to saturation (10^{-7} M) resulted in an increase of I_{Ca} amplitude from 120 to 530 pA. Upon switching to a solution containing horse serum (HS, dilution 1 : 100 v/v) in addition to the β-adrenergic agonist, I_{Ca} was reduced to about the control level. This effect was reversed after returning to an HS-free solution. In fact, responsiveness to the β-agonist continuously declined, a phenomenon that was seen to various extents in different cells. This possibly reflects desensitization and/or diffusional loss of soluble components of the signal transduction cascade. The reduction of I_{Ca}-amplitude by the 100-fold dilution of HS corresponded to the effect seen during superfusion with ACh-containing solution ($2 \cdot 10^{-6}$ M). There was no inhibitory effect of atropine (10^{-6} M) on HS-induced reduction of β-receptor-stimulated I_{Ca}, whereas the response to ACh was completely antagonized. This clearly suggests that muscarinic receptors are not involved in

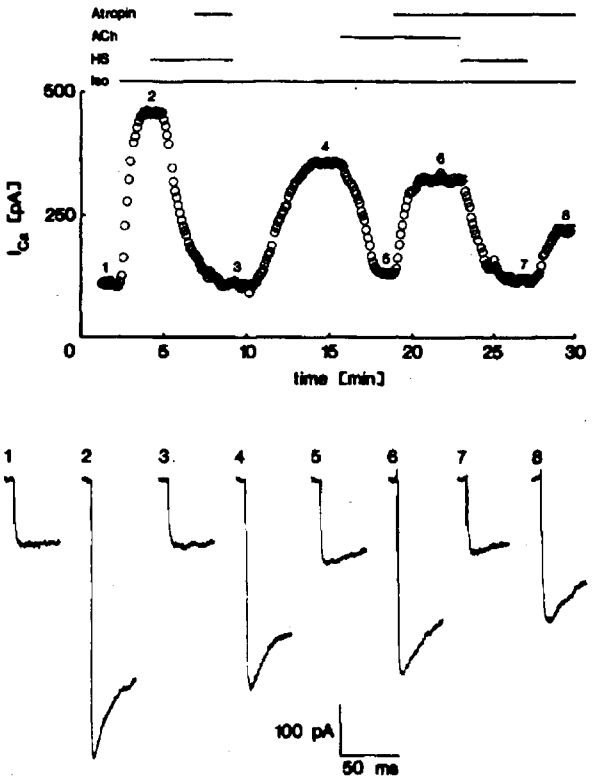


Fig. 1. *Upper panel* Stimulation of Ca^{2+} current (I_{Ca}) by isoprenaline (*ISO*, 10^{-7} M) and antagonizing effects of either horse serum (*HS*, 1 : 100 dilution) or acetylcholine (*ACh*, $2 \cdot 10^{-6}$ M). The latter effect is blocked by *atropine* (10^{-6} M), which does not affect inhibition by HS. In this and the subsequent figures I_{Ca} amplitude has been plotted against time of the experiment. The time $t = 0$ corresponds to the time at which whole cell recording started. In the *lower panel* individual current traces (leak subtracted) are shown. I_{Ca} was elicited by step depolarizations to + 5 mV from a holding potential of −50 mV at a frequency of 0.15 s^{-1}. For clarity the part of the traces recorded upon repolarization has been omitted. As a p/1 procedure was applied for leak subtraction, and no tetrodotoxin was used for blocking I_{Na}, a large Na current would have been added which is always elicited by clamping back from −105 to + 50 mV. In this and the subsequent figures the numbers on the individual traces refer to the corresponding times labelled in the plot of I_{Ca} against time

the inhibition of β-receptor-stimulated I_{Ca} by serum. A complete lack of inhibition of the serum effect by atropine was found in all eight myocytes studied in this respect, regardless of whether atropine was added during superfusion with serum-containing solution, or was already applied before serum. Both conditions are illustrated in Fig. 1. The current recordings in the lower panel are representative for the points of time indicated in the upper panel. As described previously [2, 14], inactivation of I_{Ca}, also with Ca^{2+} as charge-carrying ion, is very slow or even absent, depending on the amplitude, in cells dialysed with a citrate-containing solution, since Ca^{2+}-dependent inactivation is removed.

The inhibitory effect of serum is limited to β-adrenoceptor-stimulated I_{Ca}. Dilution of 1 : 100 or higher that consistently caused a 100% inhibition of β-adrenoreceptor-stimulated I_{Ca} had no effect on basal I_{Ca} ($n = 5$). A representative result is illustrated in Fig. 2. In this myo-

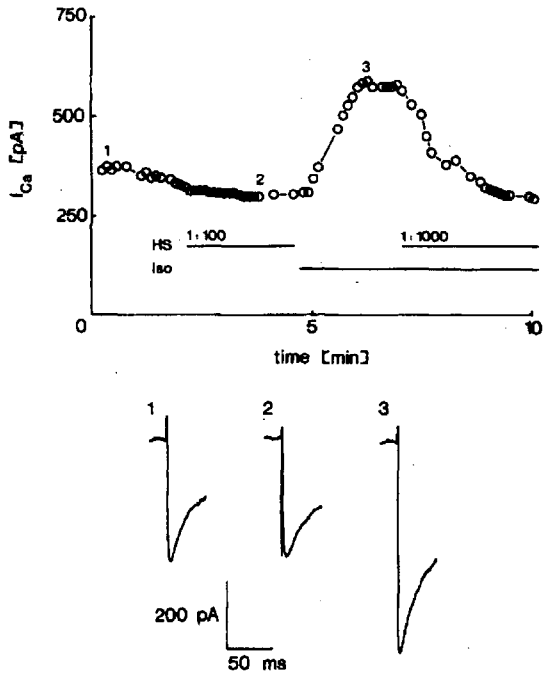


Fig. 2. Serum does not affect basal I_{Ca}. I_{Ca} amplitude is plotted against time. Solutions were superfused as indicated by the *horizontal bars*

cyte a 1000-fold dilution of HS completely reversed the effect of isoprenaline, whereas a tenfold higher concentration of HS (1:100 dilution) did not affect basal I_{Ca}.

The effect of serum is concentration dependent. Figure 3 A illustrates the effect of three different solutions (1:5000, 1:1000 and 1:500) on β-receptor-stimulated I_{Ca} recorded from a single myocyte. Already with the highest dilution the stimulated fraction of I_{Ca} was reduced by 31% in that myocyte. As shown above, a dilution of 1:100 always caused complete inhibition of that fraction of I_{Ca}. A concentration/response relationship is illustrated in Fig. 3 B. A half-maximal effect occurred at a dilution of approximately 1:1000. This closely corresponds to the potency of diluted HS to activate $I_{K,ACh}$ in the same type of cells, as described previously [1]. The effect of sera on β-receptor-stimulated L-type I_{Ca} is not limited to myocytes kept in culture for certain periods of time. It is also seen, to approximately the same extent, in freshly isolated guinea-pig atrial (n = 5) and ventricular (n = 4) cells (not illustrated).

Reduction of the amplitude of β-receptor stimulated I_{Ca} by muscarinic agonists is primarily brought about by inhibition of AC [5, 9]. Perfusion or dialysis of a cell with a high concentration of the cyclic nucleotide stimulates I_{Ca} via the same final mechanism as β-adrenergic stimulation. The current, however, becomes insensitive to regulatory inputs acting via either stimulation or inhibition of AC [5, 24]. Dialysis of a myocyte with a solution containing cAMP can thus be used to test whether the anti-adrenergic effect of serum proceeds at the level of cAMP synthesis. A representative example of such an experiment, using a pipette solution supplemented with cAMP (100 μM), is illustrated in Fig. 4. Immediately

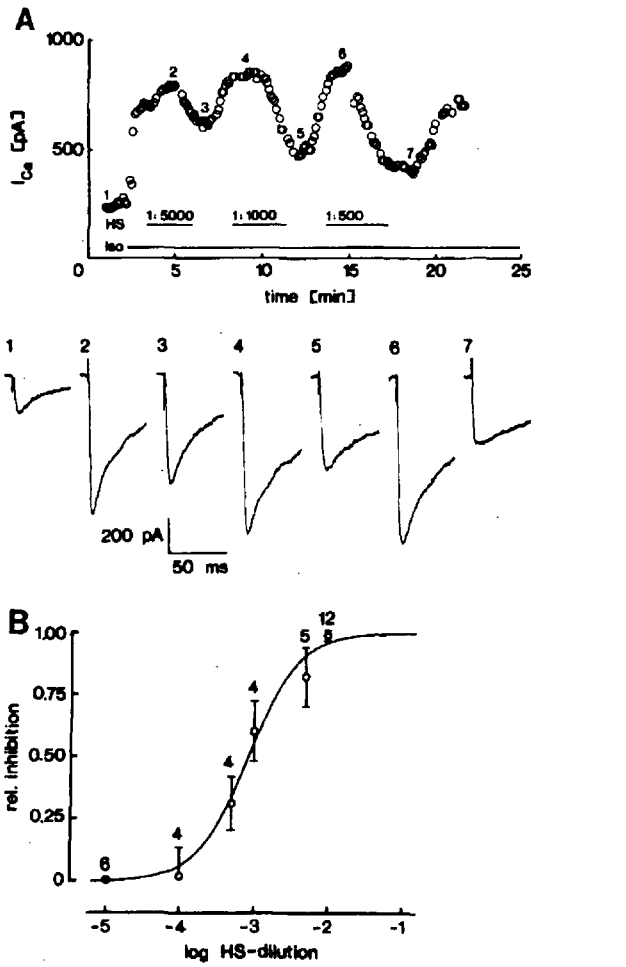


Fig. 3 A, B. Anti-adrenergic effect of serum is concentration dependent. **A** Three different dilutions of HS tested on a single myocyte. **B** Dilution/response curve summarizing the data from 12 cells in which at least two concentrations were tested (means ± SEM). The *curve* was fitted to the experimental data using an dilution producing a half maximal effect ("EC_{50}") of $9 \cdot 10^{-4}$ and an "Hill coefficient" of 1.1

after the whole-cell configuration was established by destruction of the membrane patch under the tip of the recording pipette, I_{Ca} amplitude increased beat for beat. The first Ca^{2+} current included in the plot was recorded about 20 s thereafter on the screen of the oscilloscope. Basal I_{Ca} in this cell was most likely even smaller. Within about 3 min I_{Ca} amplitude increased by 125%. Superfusion of the cell with isoprenaline-containing solution resulted in no further increase. After switching to a solution containing a 1:100 dilution of HS I_{Ca} amplitude (i. e. basal plus stimulated) was reduced by about 8%. Rundown of the current in this experiment was taken into consideration for this estimate. In a total of four experiments on cAMP-loaded cells the reduction of total current by serum in the presence of isoprenaline was 8–17% compared with the 42–78% in cells not loaded with the cyclic nucleotide. The data on cAMP-loaded cells are estimates. As I_{Ca} amplitude started to increase from the very beginning of an experiment of this type, the stimulated fraction of I_{Ca} could not be de-

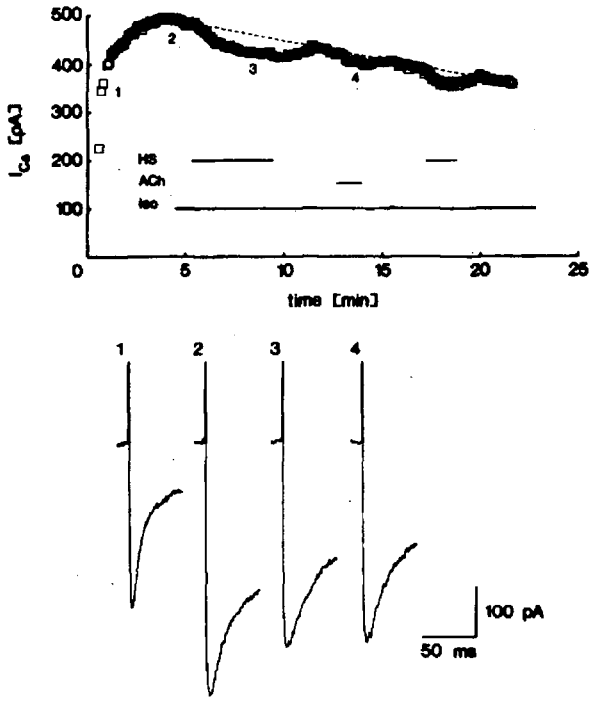
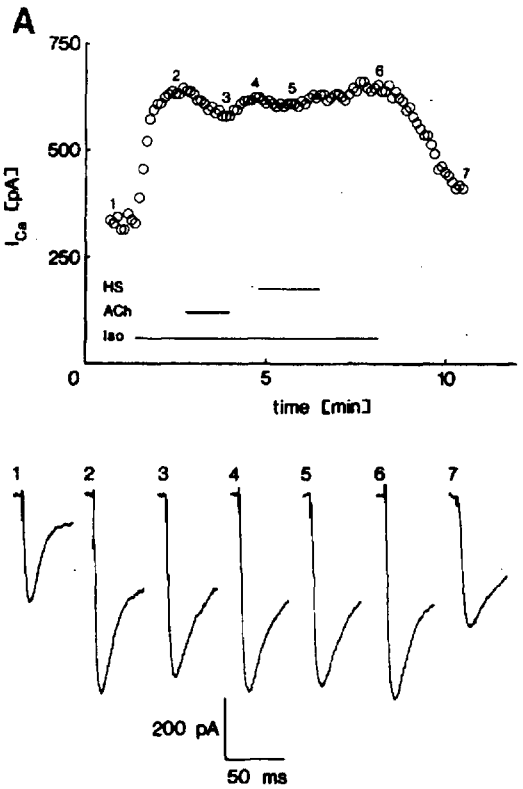


Fig. 4. Effects of HS and ACh on I_{Ca} of a myocyte loaded with cyclic adenosine monophosphate (cAMP, 10^{-4} M). Pipette filling solution was supplemented with cAMP (10^{-4} M). The amplitude of the first I_{Ca} (at t = 20 s) plotted in the top panel was read from the screen of the oscilloscope



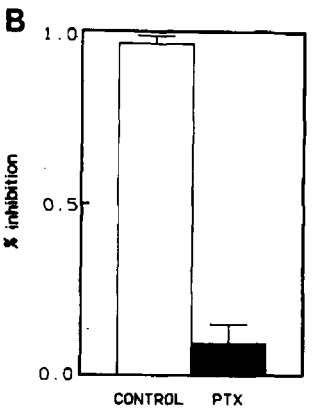


Fig. 5 A, B. Pertussis toxin (*PTx*) inhibits the effect of HS on β-receptor-stimulated I_{Ca}. **A** I_{Ca} as a function of time in a cell from a culture incubated with PTx-containing solution (1 µg/ml for about 17 h). **B** Summarized data from eight PTx-treated cells compared with untreated (control) cells from the same batches (n = 6)

termined unambiguously. Even with the additional error brought about by rundown of I_{Ca} – as shown in Fig. 4 these data clearly show that serum-induced inhibition of β-receptor-stimulated I_{Ca} is predominantly mediated by a reduction of cAMP synthesis, i. e. by the same pathway that is triggered by binding of an agonist to the muscarinic receptor.

The inhibition of β-receptor-stimulated cardiac I_{Ca} by ACh has been shown to be abolished by pretreatment of the myocytes with PTx [9, 16], which causes ADP ribosylation of the α-subunit of a distinct class of G-proteins, including G_i. Figure 5 A illustrates a representative experiment demonstrating that the signalling pathway activated by the serum factor is absent in cells treated with the toxin (1 µg/ml; 15–24 h). In this cell a slight (< 20%) reduction of I_{Ca} amplitude (stimulated component) was recorded during superfusion with ACh-containing solution in the presence of isoprenaline. No effect, however, of a 1:100 dilution of serum was observed. Comparable results were obtained in eight different PTx-treated myocytes from four batches of cells. The data are summarized in Fig. 5 B.

As shown previously for activation of $I_{K,ACh}$, the effect of serum is not limited to a certain donor species or supplier of serum. Qualitatively similar effects as those described above for HS (H 6762, Sigma) were obtained with sera from two different human donors and from the same guinea-pigs from which the myocytes had been isolated. Furthermore, the factor is not removed by dialysis. Results from four experiments, where the effects of HS, dialysed HS and the dialysing fluid were compared, are summarized in Fig. 6. The lack of a significant effect of dialysis (22–26 h against a 100-fold greater volume of extracellular solution 4° C, 10 kDa molecular weight cut-off membranes) suggests the active factor to be either a protein or to be firmly associated with a protein. Using Sephadex chromatography in order to further characterize the factor, a broad peak of activity was found in the range of 50–70 kDa, i. e. it co-purified with albumin, the most abundant protein in serum. When tested in a range of concentrations corresponding to a

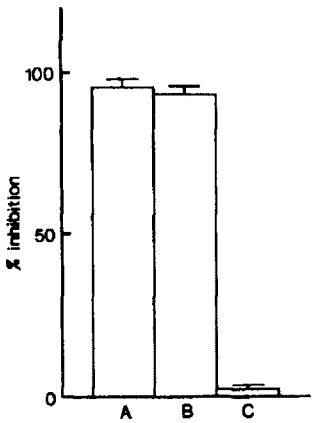


Fig. 6 A–C. Dialysis does not remove the active serum factor. **A** Control HS; **B** Dialysed HS (22–26 h against a 100-fold greater volume of extracellular solution); **C** dialysing fluid. Each bar represents four measurements ± SEM

100-fold serum dilution, different samples of purified fatty acid free albumin, however, had no effect on β-receptor-stimulated I_{Ca} (not shown).

Discussion

In mammalian cardiac muscle different receptors have been shown to mediate inhibition of AC, such as e. g. the muscarinic (M2) or purinergic (A1) receptors [5, 9, 25]. The common signalling pathway initiated by these receptors is activation of an inhibitory, PTx-sensitive G-protein (G_i) by guanosine triphosphate/guanosine diphosphate exchange on its α-subunit (see [8, 20] for review). In the present study we show that highly diluted sera contain a factor that, most likely via G_i, inhibits β-receptor-stimulated I_{Ca}. The signalling pathway seems to be identical to that initiated by activation of muscarinic or purinergic (A1) receptors, i. e. it is mediated via inhibition of AC.

We can exclude a contribution of muscarinic receptors to the effect of serum. Again, consistent with a previous study on activation of $I_{K,ACh}$ by diluted sera [1], the classic muscarinic antagonist atropine in a concentration corresponding to about 100 times the dissociation constant of this antagonist did not influence the serum effect. As serum-evoked inhibition of β-receptor-stimulated I_{Ca} was half maximal for a dilution of about 1:1000, the contribution of other receptor ligands, such as adenosine or known regulatory peptides, can be excluded. This is further supported by the finding that the factor was not dialysable. Nevertheless, the active principle has to be present in serum in a completely saturating concentration. It seems unlikely, therefore, that the serum factor plays a role in terms of normal physiological regulation of cardiac contractility and frequency. If this factor acted in the intact organ with the same potency as shown here for isolated myocytes, vagal regulation of the heart would be completely abolished. Presumably, under physiological conditions, i. e. in the intact organ, access of the factor to the myocyte or the putative receptor on its surface is prevented. In this respect it is conceivable that the present findings represent an artifact related to isolated myocytes, which are nowadays a standard preparation in basic cardiac research. An alternative hypothesis is that the factor might be produced or activated by the process of blood clotting in which case it may still be of some pathophysiological relevance.

Although the nature of the active principle in serum has to be further delineated, it seems to share some properties in common with a serum component described recently that potently activates an inositol triphosphate response in *Xenopus* oocytes [22] and the neuronal PC12 cell line [4]. In these studies an albumin fraction was identified as the active principle. This fraction, termed "activated albumin", requires certain lysolipids and presumably is formed upon blood-clotting [21]. Whether or not the serum factor described here and the factor(s) described by Tigyi and Miledi [21] are identical is under current investigation.

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