Sulfonylurea receptor ligands modulate stretch-induced ANF secretion in rat atrial myocyte culture

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Sulfonylurea receptor ligands modulate stretch-induced ANF secretion in rat atrial myocyte culture. Am J Physiol Heart Circ Physiol 278: H2028–H2038, 2000.—Stretch-induced atrial natriuretic factor (ANF) secretion was studied in cultures of neonate atrial appendage myocytes. Stretch, applied for 40 min by hypotonic swelling, increased the mean area of 44 individually imaged myocytes by 4.8–8.8% (P < 0.0001) at 6 min and by 2.3–6.2% (P < 0.05) at 35 min. Stretch increased immunoreactive ANF release by 42% (P < 0.05) from a baseline of 315 pg/ml. The ATP-sensitive K⁺ (KATP)-channel blocker tolbutamide (100 µmol/l) increased the stretch-stimulated release to 84% (P < 0.01) over baseline, whereas lower concentrations (1, 10, and 30 µmol/l) had no stimulatory effect. The KATP-channel opener diazoxide (0.1, 1, 10, 30, and 100 µmol/l) inhibited stretch- plus tolbutamide-stimulated ANF release in a concentration-dependent manner, with IC₅₀ = 2.2 µmol/l, although 100 µmol/l diazoxide did not reduce the increase in mean cell area. The stretch-stimulated KATP current, monitored in 82 whole cell recordings with sulfonylurea receptor (SUR) ligands, was inversely correlated with the stretch-induced ANF release (r² = 0.79, P < 0.0001). In the absence of stretch, the KATP current had no relationship with baseline ANF release, and baseline ANF release was not affected by the KATP-channel modulators. The results show that SUR ligands that open KATP channels inhibit stretch-induced ANF release in atrial myocytes, in correlation with the stretch-activated KATP current. The subcellular site of action of the SUR ligands—plasmalemma or intracellular organelles—remains to be determined.

ATRIAL NATIONIURETIC FACTOR; ATRIAL NATIONIURETIC FACTOR REGULATION; DIAZOXIDE; ADENOSINE 5'-TRIPHOSPHATE-SENSITIVE POTASSIUM CHANNELS; TOLBUTAMIDE

THE CARDIAC ATRIA secrete the 28-amino acid peptide atrial natriuretic factor (ANF), a potent endogenous diuretic and vasodilator (9). Multiple stimuli cause ANF secretion, including atrial stretch, hypoxia, cardiac sympathetic activation, norepinephrine, and endothelin-derived factors such as endothelin (for review, see Refs. 28 and 33). Although signaling for ANF secretion in response to norepinephrine and endothelin is relatively well established, the mechanisms of stretch-induced ANF secretion are less well understood. Endothelin mediates in part the stretch-induced ANF release in isolated atria (30) and in vivo (2, 22), whereas endothelin-independent mechanisms intrinsic to the atrial myocytes also contribute (15).

Does ion flow through stretch-activated channels trigger ANF secretion? Several types of such channels exist in the atria, including KATP (35), chloride (34), and cation channels (19). Although experiments with ion substitution have remained inconclusive so far, pharmacological modulation of KATP channels potently modulates the stretch-stimulated ANF secretion (20, 37). KATP-channel openers inhibit the stretch-induced ANF secretion in isolated rat hearts (37), although they have no effect on stretch-stimulated ANF secretion in isolated atria (20). Conversely, KATP-channel blockers increase stretch-induced ANF release in isolated hearts (37) but inhibit this release in isolated atria (20). Thus published studies have come to different conclusions on the role of KATP channels in stretch-stimulated ANF release. Some authors favor the view that opening KATP channels by stretch causes hyperpolarization, decreased Ca²⁺ influx, and therefore increased ANF release (20). Alternatively, stretch operates through a different, unknown mechanism to cause ANF secretion, and simultaneously opens KATP channels to limit this secretion in a negative feedback manner (37). The cellular targets of the KATP-channel modulators are not established. Possibilities include the atrial myocytes, endothelial cells, cardiac nerve endings, or ventricular myocytes.

For these reasons, the effect of KATP-channel modulators was studied in atrial appendage myocyte cultures, without interference of endothelial cells, nerve endings, or mechanical effects of the ventricles. Stretch by hypotonic swelling, a reproducible, potent stimulus (15), was used and quantified by imaging of individual myocytes. Patch-clamp recordings of the whole cell KATP current and membrane potential checked the combined effects of stretch and of KATP-channel modulators. The results show that 1) the pharmacological or metabolic opening of KATP channels does not reduce the swelling-induced increase in cell volume (stretch), 2) the KATP-channel openers inhibit the stretch-stimulated ANF release, and 3) the inhibition of stretch-
stimulated ANF release correlates with the magnitude of the stretch-activated K$_{ATP}$ current.

METHODS

Cell Cultures

Atrial appendage cells from neonate rats (2–4 days old; Sprague-Dawley, Dominion, and RCC, Fullinsdorf, Switzerland) were cultured as described previously (23) with the use of enzymatic dispersion (0.2% trypsin, Worthington) of atrial appendages minced into fragments of 1–2 mm$^3$. For ANF release studies, a total of 37 culture series were performed by plating 4.30 ± 0.24 × 10$^6$ cells/well in 24-well plates (Primaria, Falcon). The culture medium consisted of DMEM: Ham’s F-12 (1:1 vol/vol), 2.5% fetal bovine serum, and 7.5% horse serum (all from GIBCO). Antibiotics included 18.9 U/ml penicillin G, 3.8 µg/ml streptomycin sulfate, 7.5 µg/ml gentamicin sulfate, and 0.3 µg/ml amphotericin B (all from Sigma). The cells were cultured for up to 4 days in a 5% CO$_2$ incubator at 37°C. Culture medium was renewed every second day.

To check the proportion of cultured ANF-positive myocytes, separate cultures on glass slides were immunostained with a rabbit anti-rat ANF antiserum (IHC 9103; Peninsula Europe, Petersborough, UK; dilution 1:1.000). The primary antibody was visualized with a Texas red-labeled goat anti-rabbit gamma-globulin (T2767; Molecular Probes Europe; dilution 1:200) for immunofluorescence imaging (Fig. 1) or the peroxidase-based Vectastain Elite ABC system (Vector Lab) for performing cell counts. Immunopositive cells of the myocyte phenotype were counted in five microscopic fields (×40 objective) in each of four cultures fixed in 4% paraformaldehyde up to eight times.

% myocytes

![Fig. 1. Atrial natriuretic factor (ANF)-immunostained cell. Inset: means ± SE of percent immunopositive cells of myocyte phenotype in 4 culture series each. See METHODS for details.](http://www.ajpheart.org/content/150/1/H2029/F1.large.jpg)

Drugs

Tolbutamide, diazoxide, and pinacidil (0.1–100 µmol/l; all from Sigma) or their vehicles were applied throughout the preincubations and experimental protocol (see below). These drugs and their concentrations were chosen because they were applied in previous experiments on isolated hearts (37); thus results from atrial appendage cells and hearts could be compared. The maximum concentrations of tolbutamide and diazoxide (100 µmol/l) are below the effective concentrations used in patients (14). Lower concentrations were also used. Stock concentrations were 40 mmol/l for tolbutamide in 95% (vol/vol) ethanol, 100 mmol/l for diazoxide in DMSO, and 100 mmol/l for pinacidil in 35% (vol/vol) ethanol and 3.5% (vol/vol) HCl. The final maximum concentrations of vehicles were ≤0.25% ethanol or =0.1% DMSO. All vehicles were coapplied in controls within each culture plate.

The A- and B-endothelin receptor antagonist bosentan was a gift from M. Clozel (8). Endothelin-1 was from Peninsula and was applied to test the effectiveness of bosentan in the culture system. The endothelin concentration (10 nmol/l) of this study was previously used in culture (23).

Experimental Protocols for ANF Release Studies

Hypotonic stretch was chosen as a stimulus, because it reliably induces ANF secretion (15) and is already known to open stretch-sensitive K$_{ATP}$ channels (35). The cultures were seeded in 24-well plates, so multiple treatments could be performed with three to six wells per group within a single culture series. The cultures were equilibrated with HEPES buffer (see below) at 37°C in an air incubator, with two incubation periods of 20–40 min before starting the experimental protocol: baseline (40 min), stretch or time control (40 min), recovery (40 min), and second recovery (40 min). Recoveries were performed because ANF release was observed to rebound in some of the experiments. The HEPES buffer (1 ml; see below) was collected on ice for ANF RIA after each incubation and was replaced with 1 ml prewarmed buffer (37°C). Hypotonic stretch of the plasma membrane was applied with 1 ml per well of extracellular HEPES buffer at an osmolality of 232 mosmol/kH$_2$O (composition in mmol/l: NaCl 100, KCl 5, CaCl$_2$ 1, MgCl$_2$ 1.5, HEPES-NaOH 10, and glucose 10, pH = 7.3; adapted from Ref. 1). This stretch was preceded and followed by incubations in isotonic buffer (same composition, osmolality adjusted to 290 mosmol/kH$_2$O with sucrose, pH = 7.3).

Five 24-well culture plates were used to test the role of the K$_{ATP}$-channel blocker tolbutamide in stretch-induced ANF release, 3 plates for establishing a concentration-response curve for tolbutamide, 18 plates for testing the role of the K$_{ATP}$-channel opener pinacidil and diazoxide, 5 plates for establishing a concentration-response curve for diazoxide, 3 plates for testing the metabolic inhibitor 2-deoxyglucose, and 3 plates for testing the role of endothelin receptors. Each test (3–6 wells/test) was thus performed at least three times and up to eight times.
The radioimmunoassay was performed similarly as described previously (23) by incubation in duplicate, for 48 h, 4× diluted 50-µl samples of the medium with 25 µl of diluted NH2-terminal specific antiserum (final dilution in tube: 1:100,000) and 25 µl of 125I-labeled rat ANF (1–28) (2,000 counts·min⁻¹·tube⁻¹; Amershams; IM.1861). Antibody-bound tracer was precipitated by goat anti-rabbit antibody and counted in a Micromedics System γ-counter. Correlation coefficients (r²) averaged 0.96 ± 0.05, the 5% and 50% displacement were 0.79 ± 0.08 and 9.8 ± 0.7 pg/tube, respectively, the intra- and interassay coefficients of variation were 5.8 ± 0.6% and 14.3%, respectively. Baselines in the culture medium ranged from 100–400 pg/ml, or 1.25 to 5 pg/50 µl of 4× diluted sample, far above the threshold of detection (0.125 to 0.25 pg/tube).

Measurements of Myocyte Volume

To check the effect of hypotonic swelling during various pharmacological treatments, cell mean area and volume were monitored on culture days 2–4 at 37°C and after a 40-min preincubation during baseline (30 min), hypotonic swelling (40 min), and one recovery period (40 min). A total of 44 myocytes were scanned repeatedly in the vertical direction on an inverted microscope (Nikon, Diaphot 200; ×100 oil objective) by recording 30 optical sections separated by 300 nm on a Princeton Instruments cooled CCD camera. The zero reference was established by focusing on a spot on top of the glass slide, and the mean area of the eighth to twelfth section was measured by Huygens deconvolution software (Iterative; Bitplane, Zurich, CH) to remove out-of-focus fluorescence, the analysis was established by focusing on a spot on top of the glass slide, and the mean area of the eighth to twelfth section was measured by Huygens deconvolution software (Iterative; Bitplane, Zurich, CH) to remove out-of-focus fluorescence, the image stack followed each fluorescence image stack. Changes in extracellular K⁺ concentrations caused a shift in the current reversal potential as predicted by the Nernst equation (4). A voltage ramp was applied over 10 s from −80 mV to +90 mV to the voltage-clamped cell, and the KATP current was measured at 50 mV (after leak subtraction) and normalized by the cell capacitance. The latter was measured by capacitance compensation on the patch-clamp amplifier (4). The membrane potential was recorded at 0 pA after completion of the ramp protocol. Pharmacological treatments were similar to those described above (see also Fig. 9).

Data Analysis

Average baseline ANF concentrations (in pg/ml) were obtained from four to six groups of four to six identically treated wells for each culture plate. To analyze the effect of stretch, each well served as its own control; ANF concentrations and myocyte volumes in test and recovery were expressed as percentage of baseline and averaged over the groups. Nonparametric two-way ANOVA for repeated measures was applied for testing significant differences relative to baseline and for testing significant differences between treatments (drug vs. vehicle). Statistical analysis was performed with the SAS software from the SAS Institute, Carey, NC. For patch-clamp experiments, recordings of the whole cell KATP current were averaged for each treatment, and differences were analyzed as above. The mean baseline KATP current was plotted vs. the mean baseline ANF secretion, and the mean stimulated KATP current (at 260 mosmol/kgH2O) was plotted as a function of the mean stimulated ANF secretion (at 232 mosmol/kgH2O). Linear least-square regressions were performed. The means were weighted by the number of experiments and the reciprocal of the variances, according to the procedure of SPSS (Chicago, Illinois).

RESULTS

Effect of KATP-Channel Modulators on the Swelling-Induced Increase in Myocyte Mean Area

Hypotonic swelling caused at 6 min a significant increase in mean cell area of 5.3% (P < 0.0001) in the vehicle controls (DMSO and ethanol groups combined),
subsiding to an increase of 2.5% ($P < 0.005$) within 35 min of stretch. There was no significant difference between ethanol- and DMSO-treated cells ($P > 0.2$). On return to isotonic buffer, the mean area returned to within 1.6% of the baseline value (Fig. 2).

The pretreatment with 2-deoxyglucose had no significant effect relative to the vehicle controls and relative to diazoxide. The pretreatment with tolibutamide or diazoxide tended to enhance the swelling-induced increase in mean area relative to the vehicle controls [8.8, 7.7, 7.3, and 7.2% for 10 µmol/l tolibutamide, tolibutamide plus diazoxide (both 100 µmol/l), 100 µmol/l tolibutamide, and 100 µmol/l diazoxide, respectively; all $P < 0.0001$]. Thus the pretreatments had either no effect relative to the vehicle controls or increased somewhat the stretch induced by hypotonic swelling.

**Effect of K$_{ATP}$-Channel Modulators on Stretch-Stimulated ANF Secretion**

Tolbutamide. Successive isotonic incubations of 40 min showed a relatively stable ANF release over the 160-min protocol (Fig. 3, left panel). Hypotonic stretch significantly increased ANF secretion by 37% (Fig. 3, right panel). In the presence of the K$_{ATP}$-channel blocker tolibutamide (100 µmol/l), hypotonic stretch increased ANF secretion by 83% (Fig. 3, middle panel), a significantly higher increase than without the tolibutamide pretreatment. This experiment was repeated five times with the same outcome.

In three other culture series, the cells were exposed to 1, 10, 30, and 100 µmol/l of tolibutamide. Stretch-induced release of ANF was doubled at 100 µmol/l tolibutamide (Fig. 4), confirming the results of Fig. 3. Lower concentrations were ineffective, with the exception that 10 µmol/l of tolibutamide reduced the stimulated release.

K$_{ATP}$-channel openers. The experiment with 100 µmol/l of tolibutamide was repeated by adding 100 µmol/l of diazoxide throughout the experiment. Diazoxide abolished the stretch- plus tolibutamide-stimulated ANF secretion (Fig. 5A, right panel). In the absence of tolibutamide, diazoxide at either 50 or 100 µmol/l also abolished the stretch-induced ANF secretion (Fig. 5B). To test another class of K$_{ATP}$-channel secretion, the above experiment was repeated with pinacidil with a similar outcome (Fig. 6).

Because plasmalemmal K$_{ATP}$ channels in cardiac myocytes are thought to be mildly or not at all sensitive to diazoxide (16), it was interesting to establish a concentration-response relationship. In three different culture series, 0.1–100 µmol/l diazoxide was added to the 100 µmol/l tolibutamide-treated cells (Fig. 7). This shows that lower concentrations are effective, as diazoxide inhibited the stretch- plus tolibutamide-stimulated ANF secretion in a concentration-dependent manner. Linear regression analysis shows an IC$_{50}$ at a log (micromolar) value of 0.35, thus 2.2 µmol/l.

2-Deoxyglucose. Metabolic stress is known to open K$_{ATP}$ channels (40). To test whether it also affects ANF...
secretion, the glucose in the extracellular buffer was replaced by 2 mmol/l of 2-deoxyglucose, a compound that blocks the glycolytic production of ATP (40). Results from three separate culture series show that stretch-induced ANF release is far less in the presence of 2-deoxyglucose compared with glucose (Fig. 8A). Addition of 100 µmol/l tolbutamide to 2-deoxyglucose-treated cells restores the ANF response, and endothelin is capable of eliciting a significant ANF response in 2-deoxyglucose-treated cells. This indicates that the effect of 2-deoxyglucose was specific and not only due to a general decrease in responsiveness of the cells. ANF baselines were not different in 2-deoxyglucose, 2-deoxyglucose plus tolbutamide, or glucose alone (see Fig. 8).

Bosentan Control

Because the presence of endothelial cells in atrial cell cultures cannot be totally excluded, we checked the possible involvement of endothelin receptors in the ANF responses. Bosentan, a type A and B endothelin receptor antagonist (8), reduced the effect of 10 nmol/l endothelin on ANF secretion by 73% (see Fig. 8B). However, it had no significant effects on stretch- or stretch- plus tolbutamide-stimulated ANF secretion (Fig. 8B). It is unlikely, therefore, that endothelin mediated the pharmacological and mechanical effects on ANF release in this culture system.

Whole Cell K<sub>ATP</sub> Current

Under control conditions, and with 4 mmol/l ATP in the pipette, a voltage ramp induces only a small leak current (trace a) during baseline in an atrial appendage myocyte (see Fig. 9B). Addition of hypotonic buffer (260 mosmol/kgH<sub>2</sub>O) activates an outward current (trace b) that is further increased by application of 100 µmol/l diazoxide (trace c) and strongly reduced by 100 µmol/l tolbutamide (trace d). In other experiments (see Fig. 9B), 100 µmol/l tolbutamide was added first (trace a) and hypotonic stretch later (trace b), with similar final effects on the K<sub>ATP</sub> current. Trace c shows that the K<sub>ATP</sub> current can be further increased by diazoxide. Figure 9A shows the mean K<sub>ATP</sub> current (mean ± SE; n = no. of recordings) for various experimental conditions. The stretch-induced K<sub>ATP</sub> current is reduced by 10 and 100 µmol/l tolbutamide treatment in a concentration-dependent manner, either in the absence or presence of 100 µmol/l diazoxide. For example, in the absence of diazoxide, 10 and 100 µmol/l tolbutamide decrease the stretch-induced K<sub>ATP</sub> current by 15.5 ± 0.8% and by 60.6 ± 9.7% (P < 0.05). To simulate the effect of 2-deoxyglucose, ATP was omitted in the pipette solution. This induced a spontaneous, large K<sub>ATP</sub> current,
even in the absence of stretch, that could be significantly reduced by 100 µmol/l tolbutamide (see 0 mmol/l ATP in Fig. 9A). With 4 mmol/l ATP in the pipette, diazoxide alone (without stretch) also induced a large $K_{ATP}$ current that could be reduced by 100 µmol/l tolbutamide. In the absence of stretch but with 4 mmol/l ATP in the pipette, $K_{ATP}$ currents were all close to zero pA/pF in the absence or presence of 100 µmol/l tolbutamide (Fig. 9A).

Opening the $K_{ATP}$ channels induces a hyperpolarization of the membrane. Figure 9C shows the saturable relationship between $K_{ATP}$ current amplitude (measured at 50 mV) and the subsequent membrane potential (measured at 0 pA). The dotted curve represents the best fit for all pretreatments and shows that the membrane potential tends towards the Nernst equilibrium potential for K$^+$. The relatively low resting potential is due in part to the patch-clamp conditions, because shortly after breaking the membrane the cells showed much more negative potentials. The cells were initially normally polarized, as indicated by the fact that they were spontaneously contracting before the patch-clamp recording (see also Ref. 4, Online Methods).

Relationship Between Stretch-Stimulated $K_{ATP}$ Current and ANF Secretion

The mean stretch-stimulated $K_{ATP}$ current density (in pA/pF) was plotted vs. the mean stimulated ANF secretion (Fig. 10A) for five experimental conditions shown in the legend to Fig. 10. The 2-deoxyglucose treatment is not included because it is not possible to duplicate in whole cell recordings. There was a significant inverse relationship between stretch-stimulated current density and stretch-stimulated ANF secretion ($r^2 = 0.79$, $P < 0.0001$; Fig. 10A) but not between $K_{ATP}$ current density (no stretch applied) and baseline ANF secretion (Fig. 10C). Similarly, with stretch applied, the membrane potential was positively correlated ($r^2 = 0.69$, $P < 0.0001$) with the stretch-stimulated ANF secretion (Fig. 10B), whereas without stretch applied, the membrane potential was not related to the baseline ANF secretion (Fig. 10D).

![Fig. 6. Inhibition of stretch-induced ANF release by $K_{ATP}$-channel opener pinacidil. Pinacidil (Pin; 50 or 100 µmol/l), tolbutamide (100 µmol/l), both, or respective vehicles were added throughout experiment. A: with tolbutamide. B: without tolbutamide. Data are means from 5 (A) or 4 (B) 24-well culture plates with 3–5 wells per group per plate. Baselines (297–491 pg/ml) are not significantly different between groups. *Significant difference relative to baseline at $P < 0.05$. **Significant difference relative to baseline at $P < 0.01$. Brackets indicate significance of differences between groups.

![Fig. 7. Concentration-dependent inhibition of stretch-induced, tolbutamide-potentiated ANF release by diazoxide. Diazoxide (0.1–100 µM), tolbutamide (100 µM), or appropriate vehicle was coapplied throughout equilibration and experimental incubations. Data were normalized relative to effect of 0 µM diazoxide, plotted on a log scale, and analyzed by linear regression ($r^2 = 0.94$, $P < 0.01$; 95% confidence intervals shown by thin stippled lines). Data are means of means ± SE from 5 separate culture plates with 6 groups of 3–4 wells per group per plate. Mean baselines (324–434 pg/ml) are not significantly different between groups.](http://ajpheart.physiology.org/.../10.220.33.5)
DISCUSSION

This study suggests, for the first time, an important role of sulfonylurea receptor (SUR) in stretch-stimulated ANF secretion in atrial appendage myocyte culture, where the effects of endothelial cells, nerve endings, and mechanical effects of the ventricle can be excluded. The results indicate that SUR ligands that open KATP channels inhibit stretch-stimulated ANF secretion. To verify that this inhibition was not due to a reduced stretch stimulus, myocyte volume changes were measured during the pharmacological pretreatments. To check that the pharmacological treatments had their intended effects, we monitored the whole cell KATP current. With 4 mmol/l ATP in the pipette, antagonism of endothelin receptors had no effect on stretch-stimulated ANF release, and tens of thousands of cocultured endothelial cells are required to modulate ANF secretion (23, 24). Regarding the stretch stimulus, it was important to show to what degree the pharmacological treatments altered the hypotonic swelling-induced volume increase. If KATP-channel openers diminished this increase, then inhibition of stretch-induced ANF release would have been simply explained by a diminished stretch. However, opening the KATP channels by 2-deoxyglucose had no effect, and diazoxide enhanced the increase in mean cell area during hypotonic swelling (Fig. 2). A potential drawback, as with all cell cultures, is that the mechanisms uncovered will need to be verified in vivo to demonstrate their physiological importance.

Did the vehicles perturb the cell volume dynamics? A concentration of 0.25% ethanol increases the medium osmolality by 40 mosmol/kgH2O. Ethanol has been shown to cause a transient (5 min) change in cell volume and prolactin secretion (29). The osmotic effects subside quickly, because ethanol equilibrates across the cell membranes. Because in our experiments the pretreatments started with the preincubations, effects of ethanol were long over when the baseline incubations began. It can be inferred that the effect of DMSO, if any, would also have dissipated, as its osmotic effect was only 14 mosmol/kgH2O. We conclude that stretch-induced ANF secretion and its modulation by pharmacological pretreatments were mediated by mechanisms intrinsic to the myocytes. Cell stretch during treatment with KATP-channel openers was equal to or greater than the stretch in the controls, and the osmotic effects of the vehicles are unlikely to have changed during the experiment.

Relationship Between Inhibition of Stretch-Induced ANF Release and Stretch-Activated KATP Current

Diazoxide exerts remarkably potent inhibitory effects on the stimulated ANF secretion, with an IC50 of 2.2 µmol/l (Fig. 7). This inhibition is obvious while the stretch is applied, although ANF appears to rebound in some cases (Fig. 5B) but not others (Fig. 5A) when the stretch is released. Pinacidil, another KATP-channel opener, had similar effects (Fig. 6). This rebound is interesting, because it may mean that stretch-induced (unknown) mechanisms are held in check, whereas KATP-channel openers are maximally activated by pharmacological agents and stretch but can cause a delayed stimulation of ANF secretion when the stretch is released. The other method of opening the atrial KATP channels was to pretreat the cells for 80 min with 2 mmol/l 2-deoxyglucose. This pretreatment is known to strongly reduce the glycolytic production of ATP (40). As was the case for diazoxide and pinacidil, 2-deoxyglucose strongly reduced the stretch-induced ANF release (Fig. 8), showing that all putative KATP-channel openers tested exert inhibitory effects on stretch-stimulated ANF release.

The patch-clamp experiments show, in parallel, that diazoxide exerts potent activating effects on the atrial whole cell KATP current. With 4 mmol/l ATP in the pipette,
a concentration of 100 µmol/l of diazoxide causes an activation of 100 pA/pF (Fig. 9). Previous experiments show that with 2 mmol/l ATP in the pipette, the half-maximal activation occurs at <10 nmol/l (4). Diazoxide was not known to have significant effects on plasmalemmal KATP channels in cardiac myocytes, although other KATP-channel openers such as pinacidil (200 µmol/l) strongly activate both ventricular and atrial plasmalemmal KATP channels in guinea pig (26). Recently, it was shown that 500 µmol/l diazoxide activates a rat ventricular KATP current of only 5 pA/pF (39). Thus rat atrial appendage myocytes are 100 to 1,000,000 times more sensitive to diazoxide than ventricular myocytes.

Tomimic in part the effect of 2-deoxyglucose, ATP was omitted in the patch pipette (Fig. 9). This also induced a large KATP current. Conversely, inhibition of the stretch-activated KATP channels with 100 µmol/l tolbutamide, as demonstrated by the patch-clamp studies (Fig. 9), potentiated the stretch-induced ANF release. The increase in mean cell area was also slightly larger but was not followed by an increased ANF response. Thus, overall, there is a significant correlation between the stretch-activated atrial KATP current and the inhibition of stretch-induced ANF release, as shown graphically in Fig. 10. A correlation does not prove, however, that a cause-and-effect relationship exists, as further discussed below.

From the results alone it is not possible to indicate the exact molecular mechanisms by which KATP-channel modulators affect the stimulated ANF secretion. The plasma membrane KATP channels are opened by bath-applied diazoxide and pinacidil and by 0 ATP in the patch pipette. However, the ANF baselines remained unaffected by diazoxide, pinacidil, and 2-deoxyglucose, as already reported for diazoxide and pinacidil in the isolated rat heart (37). Thus the conventional view that changes in cellular polarization and Ca2⁺ influx through voltage-dependent Ca2⁺ channels would alter the baseline hormone secretion, as reported for insulin (25), apparently does not apply to ANF. It remains possible that changes in cell polarization affect the stimulated secretion of ANF.
ANF release, although the mechanism involved is not yet known.

Because Ca$^{2+}$ appears to play a negative role in stretch-stimulated ANF secretion (15), opening the plasmalemmal K$_{ATP}$ channels should cause relief of Ca$^{2+}$-mediated inhibition of ANF release. However, the contrary situation was the case, because stretch-stimulated ANF secretion was clearly inhibited by all types of K$_{ATP}$-channel openers. Thus it is unlikely that plasmalemmal K$_{ATP}$ channels inhibit the ANF release by means of a hyperpolarization-induced decrease of Ca$^{2+}$ influx. It remains possible that plasmalemmal K$_{ATP}$ channels inhibit as yet unknown stretch-stimulated second-messenger mechanisms. As long as these second messengers are not identified, this view cannot be tested.

Could a time delay or different threshold (stretch) for activation of K$_{ATP}$ channels and stimulation of ANF release explain the present data? There seems to be no time delay between opening of K$_{ATP}$ channels and stimulation of ANF release, because stretch, within minutes, opens K$_{ATP}$ channels in patch-clamped cells (Ref. 35 and Fig. 9) and activates ANF release from isolated atria (e.g., Ref. 37). ANF release in culture is usually measured over longer intervals for technical reasons (assay sensitivity), but not because ANF release is delayed. If opening of K$_{ATP}$ channels preceded and caused the ANF release, then diazoxide and pinacidil should cause increased ANF secretion. This is not the case (see Figs. 3–7). Graded stretch proportionally increases the ANF secretion up to a saturation point (37) and increases the open probability of K$_{ATP}$ channels (35). Both relationships are positive and monotonic, and a different threshold, even if it existed, could not explain a negative correlation.

Could intracellular organelles (27) such as secretory vesicles (3) or mitochondria (12, 13) be involved? Recently, pancreatic secretory vesicles were shown to fuse with the plasmalemma in response to 100 µmol/l tolbutamide (3). The proposed mechanism was that tolbutamide activates a secretory vesicle chloride channel, causing vesicle swelling, a prerequisite for fusion with the plasmalemma. Diazoxide could block this action. It is not inconceivable, therefore, that the K$_{ATP}$-channel modulators may act directly on the secretory vesicles in atrial myocytes as well, an interesting concept that remains to be verified. Alternatively, diazoxide and pinacidil could have activated mitochondrial K$_{ATP}$ channels, as shown in ventricle (12), although the pharmacology of atrial mitochondrial K$_{ATP}$ channels is not yet known. Another argument possibly implicating mechanisms other than the plasmalemmal K$_{ATP}$ channels is that 10 µmol/l tolbutamide had minor effects on the stretch-stimulated plasmalemmal K$_{ATP}$ current density (Fig. 9), yet inhibited stretch-stimulated ANF secretion (Fig. 4).

Resolution of Apparent Discrepancies

Although in support of our previous work (37), this study is in apparent conflict with another report suggesting that closure of K$_{ATP}$ channels by glibenclamide inhibits stretch-stimulated ANF secretion and that pinacidil has no effect (20). Three major differences
could explain these results. First, isolated atria were used (20), where endothelin plays a major role in stretch-stimulated ANF secretion (30). The site of action of glibenclamide and pinacidil thus also included the endothelial cells, making the data interpretation more difficult. Also, externally perfused atria may be in an ischemic state, rendering the myocytes less sensitive to sulfonylurea (11). Second, supraphysiological increases in atrial pressure (2–6 mmHg) were applied (20). In rat, atrial pressures above 1.5 mmHg are considered supraphysiological (21). Overstretching the atria could interfere with the actin filaments, known to alter the sensitivity to glibenclamide (6), and a maximum activation of the K_\text{ATP} channels would render the ANF release insensitive to K_\text{ATP}-channel openers. And third, the lipophilic glibenclamide (10) at concentrations of 100 µmol/l (20) is known to have nonspecific effects, such as on inward rectifier K^+ current (I_{K1}) in the guinea pig atrium (31) and on plasma membrane lipids in rat pancreatic β-cells (5). Tolbutamide at 100 µmol/l, used in our studies, does not have these nonspecific effects; the culture system safely excludes interference of endothelin; and the stretch applied appears modest, as was the atrial pressure (1–1.5 mmHg) in the isolated heart (37).

Perspectives

In conclusion, the results suggest that the SURs of neonatal rat atrial appendage myocytes potently modulate the stretch-stimulated ANF secretion. The results show that K_\text{ATP}-channel openers inhibit the stretch-stimulated ANF release. Conversely, tolbutamide potentiates the stretch-stimulated ANF release at a concentration that half-maximally inhibits the stretch- or diazoxide-activated atrial plasmalemmal K_\text{ATP} current. Neither K_\text{ATP}-channel opener affects the baseline ANF release, suggesting mechanisms distinct from those postulated for insulin secretion. Together with other work discussed above, this study leaves us with five questions. 1) Do diazoxide and sulfonylureas and possibly endosulfine (36) target SUR on the plasmalemma or on intracellular organelles to modulate the stimulated ANF secretion? 2) Do SUR ligands affect the function not only of inward rectifier K^+ channel but also of other effectors? 3) Is the high sensitivity of the atrial-stimulated ANF release to diazoxide suggestive of a novel cardiac plasmalemmal K_\text{ATP}-channel subtype (4), or does it imply an activation of mitochondrial K_\text{ATP} channels (12)? 4) Can results from neonate atrial appendage myocytes be extended to adult myocytes and to in vivo studies? 5) Can atrial SUR help explain the complex link between diabetes mellitus, hyperglycemia, and altered regulation of ANF secretion (7)?

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