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Isoproterenol stimulates rapid extrusion of sodium from isolated smooth muscle cells

(Na⁺,K⁺-ATPase/fluorescent digital imaging microscopy/β-adrenergic receptors/cyclic AMP)

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ABSTRACT **β-Agonists cause an inhibition of contractility and a transient stimulation of Na^+/K^+ pumping in smooth muscle cells of the stomach from the toad *Bufo marinus*. To determine if the stimulation of Na^+/K^+ pumping causes changes in intracellular $[Na^+]$ ($[Na^+]_i$) that might link Na^+ pump stimulation to decreased Ca^{2+} availability for contraction, $[Na^+]_i$ was measured in these cells with SBFI, a Na^+-sensitive fluorescent indicator. Basal $[Na^+]_i$ was 12.8 ± 4.2 mM (n = 32) and was uniform throughout the cell. In response to isoproterenol, $[Na^+]_i$ decreased an average of 7.1 ± 1.1 mM in 3 sec. Since this decrease in $[Na^+]_i$ could be completely blocked by inhibition of the Na^+ pump, or by blockade of the β-receptor, $[Na^+]_i$ reduction is the result of occupation of the β-receptor by isoproterenol and subsequent stimulation of the Na^+ pump. 8-Bromoadenosine 3′,5′-cyclic monophosphate and forskolin mimicked the effect of isoproterenol, indicating that the sequence of events linking β-receptor occupation to Na^+ pump stimulation most likely includes activation of adenylate cyclase, production of cAMP, and stimulation of cAMP-dependent protein kinase. The decrease in $[Na^+]_i$ is sufficiently large and fast that it is expected to stimulate turnover of the Na^+/Ca^{2+} exchanger in the Ca^{2+} extrusion mode, thereby accounting for the observed linkage between stimulation of the Na^+/K^+ pump and inhibition of contractility in response to β-adrenergic agonists.**

The β-agonist isoproterenol (ISO) inhibits contraction in smooth muscle cells, but the subcellular events responsible for this are not fully understood. It is known that roughly 50% of the relaxant effect of ISO can be blocked by preequilibrating smooth muscle tissue with ouabain, which has led to the suggestion that stimulation of the Na^+/K^+ pump is involved in ISO's mechanism of action (1). This Na^+ pump hypothesis has been tested using radioisotope flux measurements of $^{24}Na^+$ and $^{42}K^+$ (1) and electron microprobe analysis of Na^+ and other monovalent and divalent cations (2). Although both approaches have suggested that intracellular $[Na^+]$ ($[Na^+]_i$) is most likely decreased in response to ISO, neither technique can measure free $[Na^+]_i$ or the magnitude of any change in free $[Na^+]_i$ in response to ISO. Yet, it has been suggested it is a decrease in the free $[Na^+]_i$ that is linked to the negative inotropic effect of β-agonists. It is thought that a decrease in free $[Na^+]_i$ would stimulate turnover of the Na^+/Ca^{2+} exchanger in its Ca^{2+} extrusion mode, thereby removing Ca^{2+} from the cell. Yet, without a reliable measure of the free $[Na^+]_i$, or the change in free $[Na^+]_i$, if any, in response to ISO, it is not possible to predict either the reversal potential of the Na^+/Ca^{2+} exchanger or how much of a decrease in free $[Na^+]_i$ would be required to shift the exchanger into Ca^{2+} extrusion mode.

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An additional limitation of radioisotope flux and electron microprobe analyses is their limited temporal resolutions and therefore their inability to accurately determine the time course of changes in $[Na^+]_i$. Yet there is evidence that β-agonists effect rapid changes in the chemistry and contractility of smooth muscle. Upon exposure to ISO, chemical assays reveal that there are significant increases in cAMP content and cAMP-dependent protein kinase activity within 2.5 and 5 sec, respectively. There is a significant reduction in contractility in 10 sec (1) and a significant decrease in free cytoplasmic $[Ca^+]_i$ within 3 sec (3). These data suggest that if ISO stimulates the Na^+/K^+ pump, and that effect is integral to its mechanism of action, then Na^+ pump stimulation may be quite rapid. With the development of the ratiometric Na^+-selective fluorescent probe SBFI (4), it has become possible to measure the free $[Na^+]_i$ in single cells. We have therefore undertaken a study to measure the free $[Na^+]_i$ in single, enzymatically dissociated smooth muscle cells isolated from the stomach of the toad *Bufo marinus* and to determine the extent and time course of changes in the free $[Na^+]_i$ in this tissue in response to β-adrenergic stimulation.

METHODS

Cells, Dye Loading, and Calibration. Single smooth muscle cells were obtained by enzymatic dissociation of the stomach of the toad *B. marinus* as described (5). Cells were maintained in amphibian physiological saline (APS), which consisted of (in mM) 109 NaCl, 3 KCl, 0.56 Na_2HPO_4, 0.14 NaH_2PO_4, 20 $NaHCO_3$, 1.8 $CaCl_2$, 0.98 $MgSO_4$, and 11.1 glucose, equilibrated with 95% O_2/5% CO_2, pH 7.4. Cells were loaded with the sodium-sensitive ratiometric fluorescent probe SBFI by incubating them at room temperature for 2–3 hr with the probe's cell-permeant acetoxymethylester derivative SBFI/AM (Molecular Probes); 2.9×10^{-9} mol of SBFI/AM [maintained as a 10 mM stock in dimethyl sulfoxide (DMSO)] was added per ml of cell suspension, cell density ≈10^6 per ml; given an average intracellular volume of 5.76 pl (6), there was, if all of the dye was trapped intracellularly and deesterified, up to 0.5 mM SBFI free acid within any given cell.

In situ calibration curves were acquired by equilibrating dye-loaded cells with appropriate mixtures of (in mM) (*i*) 130 NaCl/10 Na*Mops/1 $MgCl_2$, pH 7.6, and (*ii*) 130 KCl/10 K*Mops/1 $MgCl_2$, pH 7.6, such that $[Na^+]$ + $[K^+]$ was constant at 130 mM (4). The cells were permeabilized with a mixture of the Na^+/H^+ ionophore monensin (10 μg/ml) and the K^+/H^+ ionophore nigericin (10 μg/ml) or with the cationophore gramicidin D (10 μg/ml); identical results were obtained with both protocols. For *in vitro* calibration curves

Abbreviations: ISO, isoproterenol; APS, amphibian physiological saline; [ ]$_i$, intracellular ion concentration; DIM, digital imaging microscope; DWM, dual-wavelength microfluorimeter; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; DMSO, dimethyl sulfoxide.

we used 1 μM SBFI free acid and the same solutions that were used for the *in situ* calibrations.

Cell Fixation and Length Analysis. A suspension of freshly dissociated cells was divided into two aliquots, an experimental and a control group. The experimental group was loaded with SBFI/AM as described above; the control group was sham-loaded by exposing them to an equivalent volume of DMSO. At the end of the loading period each group was subdivided. One subdivision was fixed with acrolein (0.1%) for determining the length of resting cells. The other subdivision was challenged with carbachol (1 μM) for 30 sec prior to fixation. Several fields of fixed cells were imaged with a 10× objective and an 8× eyepiece using our digital imaging microscope (DIM) and the length of the long axis of the first 25 cells viewed was determined as described (7).

Fluorescence Microscopy. Our DIM consisting of a Zeiss-IM-35 microscope equipped for epifluorescence has been described in detail elsewhere (8). A drop of the dye-loaded cell suspension was settled in the cell chamber (volume = 1 ml) for 30 min prior to analysis to allow the cells to settle and to allow as complete a deesterification of cell-trapped dye as possible. Images were acquired with a 1-sec exposure. Following background subtraction and correction for variation in the illumination intensity across the field of view, individual pixels of an image were selected for analysis if they contained significant cellular fluorescence (9). Criterion for selection of a pixel was an intensity ≥3 standard deviations above the mean grey level of the background; these pixels had a signal:noise level ≥10. Ouabain, carbachol, and ISO were delivered to cells by applying pressure to the back end of a micropipette with a Picospritzer II (General Valve, Fairfield, NJ).

For following changes in $[Na^+]_i$ on a rapid time scale we used our dual-wavelength microfluorimeter (DWM), details of which can be found in Yagi *et al.* (10). For experiments in the DWM, the cell chamber was modified to allow perfusion (volume ≈ 0.5 ml), and a complete change of the solution in the chamber took about 1 sec.

RESULTS

Fig. 1*A* shows images of a SBFI-loaded cell at rest in APS and an *in situ* calibration that was acquired from the same cell. The ratio image, displayed in pseudocolor, indicates no nuclear/cytoplasmic Na^+ gradient, within the 1 mM resolution of the measurement, and the dye calibrated uniformly throughout the cell. Fig. 1*B* compares the calibration of SBFI free acid with an *in situ* calibration. The *in situ* calibration is obviously quite different from the *in vitro* calibration, and the *in situ* calibrations were generally different on different days. Conversion of ratio measurements into measurements of $[Na^+]_i$ therefore depended on an *in situ* calibration every day that experiments were performed, and $[Na^+]_i$ was calculated from the equation for the rectangular hyperbola that was fitted to the *in situ* calibration curve.

The $[Na^+]_i$ of the resting cell shown in Fig. 1*A* was 16 mM. In 32 cells examined in the DIM and DWM, the average $[Na^+]_i$ was 12.8 ± 4.2 mM (mean ± SD) and the values ranged from 6 to 23 mM. This mean value is very close to the K_m for Na^+ of the Na^+/K^+ pump of toad kidney, 12.3 mM (11).

Cells were studied individually, on either the DIM or the DWM, and screened for their ability to reversibly contract in response to the local application of 1 μM carbachol prior to use. This screening was essential as many cells loaded with SBFI became nonresponsive to contractile agonists. This effect of SBFI is demonstrated in Fig. 2. The resting length of cells that were sham-loaded was 180 ± 11.6 μm and the contractile agonist carbachol caused a significant ($P < 0.05$) decrease in the length of these cells (125 ± 8.6 μm). The resting length of SBFI-loaded cells was somewhat, though not significantly, shorter than the resting length of the control group (162 ± 9.5 μm), and although some SBFI-loaded cells did contract in response to carbachol, the mean decrease in length for the population was not significant (143 ± 8.7 μm). This effect of SBFI probably stems from the fact that SBFI

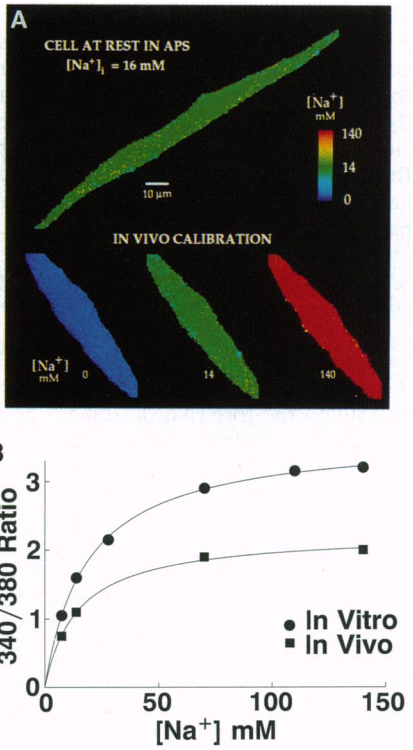

FIG. 1. (*A*) Image of the distribution of free Na^+ in a single smooth muscle cell at rest in APS and the *in situ* calibration recorded from the same cell. The ratio image is pseudocolored. For calibration the cell was exposed to monensin (10 μg/ml) and nigericin (10 μg/ml) and equilibrated with the indicated Na^+ concentration. (*B*) Comparison of the *in vitro* and *in vivo* calibration of SBFI. *In vitro* calibration was acquired on the stage of the DIM using 1 μM SBFI free acid. The *in vivo* calibration was acquired from a cell loaded with SBFI/AM and permeabilized with monensin (10 μg/ml) and nigericin (10 μg/ml). Curves were drawn by fitting a rectangular hyperbola through the points ($r > 0.98$).

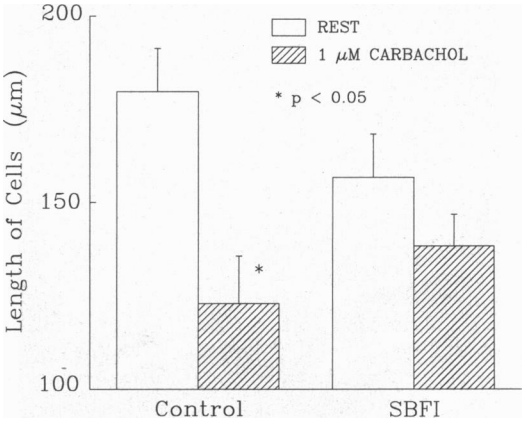

FIG. 2. Comparison of cell lengths, for cells that were sham-loaded (Control) and cells that were loaded with SBFI, before and after exposure to 1 μM carbachol for 30 sec. For each group, the length of 25 cells was measured, and the vertical bar represents the mean length of the cells in that group ± SEM. Carbachol produced a significant reduction in the length of cells that were sham-loaded but not in cells that were loaded with SBFI. Although SBFI-loaded cells at rest were somewhat shorter than sham-loaded cells at rest, this difference was not statistically significant.

is a crown ether (4) and crown ethers are known to have profound positive and negative inotropic effects on cardiac and respiratory smooth muscle, with no clear structure–function relationship among members of this family of compounds (12). Although the mechanism of their inotropic effects is not known, these results demonstrate that cells loaded with SBFI must be individually screened prior to experimentation when using SBFI. This prevents the use of cell suspensions, or other populations of cells, when using this dye, and therefore only cells that gave a vigorous reversible contraction to carbachol, similar to that seen in cells prior to SBFI loading, were used for further experimentation.

Fig. 3*A* shows images of a dye-loaded cell that were acquired using the DIM and demonstrates the effect of ouabain and ISO on the free $[Na^+]_i$. Images acquired at the isosbestic wavelength, 360 nm, demonstrate the nonuniform dye distribution. The basal $[Na^+]_i$ in this cell was 10 mM, and 10 min after the local application of 1 mM ouabain the $[Na^+]_i$ in this cell rose to 15 mM, consistent with the known effect of ouabain to inhibit the Na^+/K^+ pump. The rise in $[Na^+]_i$ was also uniform throughout the cytosol and the nucleus. Measurement at the isosbestic wavelength indicates that the dye did not redistribute during this time; there was simply an increase in fluorescence intensity at 340 nm. Fig. 3*B* shows the response of a cell to the local application of 100 μM ISO, a concentration at the peak of ISO's dose–response curve. The pseudocolored ratio image shows that the basal $[Na^+]_i$ in this cell was 11 mM. Three minutes after the local application of 100 μM ISO, $[Na^+]_i$ had fallen to 8 mM. The decrease in $[Na^+]_i$ in response to ISO was again uniform throughout the cytosol and nucleus. Though the DIM provides measurements of $[Na^+]_i$ with high spatial resolution (about 0.2 μm), the temporal resolution, limited by the time required to acquire an image at each of the excitation wavelengths and to change the excitation filter, is poor. To examine the response with greater temporal resolution, we therefore switched to the DWM, which provided us with $[Na^+]_i$ measurements every 50 msec.

Fig. 4*A* shows a typical response of a cell in the DWM to superfusion with 100 μM ISO. There was a rapid and highly significant decrease in $[Na^+]_i$. The average time to establish a new steady state in response to ISO was 3 sec, and the average decrease in $[Na^+]_i$ was 7 mM (Table 1). After

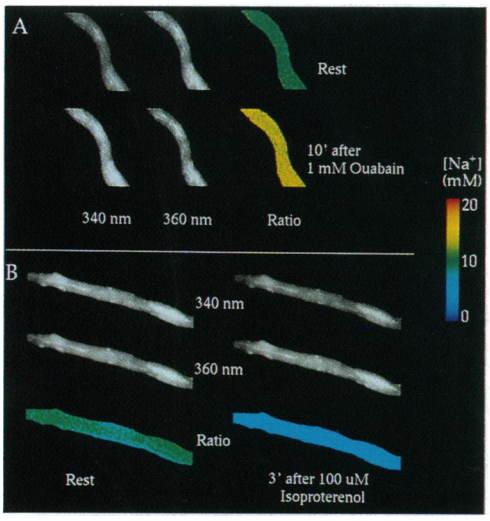

FIG. 3. Effect of ouabain and ISO on $[Na^+]_i$ in single smooth muscle cells measured on the stage of the DIM. The grey scale images were acquired at the indicated excitation wavelengths; fluorescence was recorded at 510 nm; the ratio image is pseudocolored. (*A*) $[Na^+]_i$ 10 min after application of 1 mM ouabain. (*B*) $[Na^+]_i$ 3 min after application of 100 μM ISO. Scale as in Fig. 1*A*.

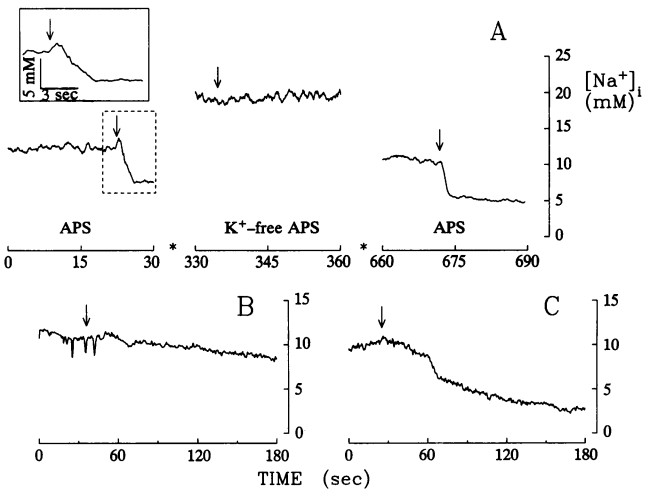

FIG. 4. High-speed records of $[Na^+]_i$ in single smooth muscle cells: Effects of ISO, $[K^+]_o$ depletion, β-adrenergic antagonists, and 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP). (*A*) Response of a single cell bathed in APS to superfusion with 100 μM ISO (application of ISO indicated by arrow), the response of the same cell to superfusion with 100 μM ISO following inactivation of the Na^+/K^+ pump by removal of K^+ from the APS for 5 min, and the reacquisition of the ISO response following reactivation of the Na^+/K^+ pump. (*Inset*) Response to ISO at higher temporal resolution. (*B*) Same cell as in *A* but the cell was preequilibrated with 10 nM of the nonselective β-antagonist pindolol for 5 min prior to challenge with 100 μM ISO. (*C*) Response of $[Na^+]_i$ in a different cell to superfusion with APS containing 5 mM 8-Br-cAMP.

replacing ISO with fresh saline, $[Na^+]_i$ returned to prestimulus levels within 5 min. The cell was not illuminated during the time $[Na^+]_i$ returned to its basal concentration so as to avoid photobleaching and possible photodamage. Up to three responses of similar magnitude have been recorded from the same cell. To test whether the decrease in $[Na^+]_i$ is due to stimulation of the Na^+/K^+ pump, or to some other mechanism, we eliminated $[K^+]_o$ from the medium. Removal of $[K^+]_o$ completely inactivates the pump and provides a more effective and rapid way to fully inactivate the Na^+ pump than ouabain in this tissue since the toad Na^+/K^+ pump is relatively insensitive to ouabain (13). While the pump was inactivated $[Na^+]_i$ rose as the passive inward sodium leak was no longer countered, and in this cell $[Na^+]_i$ reached 21 mM in 5 min. Under these conditions the drop in $[Na^+]_i$ previously seen in the same cell in response to ISO was completely abolished. Similar results were observed in every cell in which this experiment was attempted. As can be seen, when

Table 1. Effects of various agents on $[Na^+]_i$ in single smooth muscle cells

Condition	Change in $[Na^+]_i$, mM	*n*
100 μM ISO	−7.1 ± 1.1*	9
30 μM forskolin	−6.9 ± 3.4†	4
5 mM 8-Br-cAMP	−9.3 ± 3.9	3
K^+-free saline‡	+10.3 ± 2.4	3
K^+-free saline‡ + 100 μM ISO	+0.6 ± 0.3§	3
10 nM pindolol‡ + 100 μM ISO	−0.8 ± 0.9§	3

The experimental manipulation is followed by the average change from rest in $[Na^+]_i$ ± SEM; *n* = number of observations.
*Experiments on DIM and DWM.
†Forskolin was dissolved in 1% DMSO and applied to the cell by the application of pressure to the back end of a pipette on the DWM. A 1% DMSO solution did not affect $[Na^+]_i$.
‡Cell was exposed to this medium 5 min prior to recording.
§The limit of resolution of either the DIM or the DWM is ≈1 mM; changes in $[Na^+]_i$ <1 mM are therefore not significant.

the K^+-deficient medium was replaced with medium again containing 3 mM K^+, the effect of ISO on Na^+ recovered. These results indicate that the Na^+/K^+ pump is the effector responsible for reducing $[Na^+]_i$ in response to ISO. These results cannot be explained by an effect of ISO to shut down passive influx of sodium since this could change $[Na^+]_i$ by only 0.1 mM in the same amount of time.†

ISO is believed to act in this tissue as in other cells, via a β-adrenergic receptor that activates adenylate cyclase through interaction with a G protein. Experiments were therefore carried out to determine if this sequence of events could explain the effect of ISO on $[Na^+]_i$ in these smooth muscle cells. The results are presented in Fig. 4 *B* and *C* and are summarized in Table 1. In every cell in which it was tried, the β-antagonist pindolol blocked the ISO-induced reduction in $[Na^+]_i$ (Fig. 4*B* and Table 1). This indicates that the reduction in $[Na^+]_i$ is mediated via a β-receptor and is not the result of a simple nonspecific effect of the drug on Na^+ handling or on the dye. 8-Br-cAMP (Fig. 4*C*), a nonhydrolyzable analogue of cAMP, and forskolin, a stimulant of adenylate cyclase, produced decreases in $[Na^+]_i$ similar to that seen with ISO (Table 1). The magnitude of the response to 8-Br-cAMP shown in Fig. 4*C* was the largest seen. The level to which $[Na^+]_i$ falls presumably reflects a balance between enhanced pump activity from ISO stimulation, the inward leakage of Na^+, and the decrease in pump activity that occurs as $[Na^+]_i$ falls, and these presumably vary somewhat between cells.

DISCUSSION

The mean free $[Na^+]_i$ of 12.4 mM that we measured in these gastric smooth muscle cells with SBFI is similar to the 10 mM measured in rat aorta with NMR spectroscopy (14), the 7.4 mM measured in guinea pig ureter with Na^+-selective microelectrodes (15), and the 13.4 mM measured in cultured rat vascular smooth muscle cells with SBFI (16). This similarity in the mean value for $[Na^+]_i$ in smooth muscle cells measured with three different techniques provides confidence in the numerical accuracy of the *in situ* calibration of the dye. The K_m of the Na^+ pump for Na^+, measured in *B. marinus* kidney, is 12.3 mM, very close to the 12.4 mM that we have measured for the free $[Na^+]_i$ in smooth muscle (11). That these values are nearly identical suggests that $[Na^+]_i$ is set to ensure maximum responsiveness of the Na^+ pump in the face of changes in Na^+ influx or efflux and, therefore, maximum stability in $[Na^+]_i$. The large difference that we have seen between the *in situ* and *in vitro* calibration curves for SBFI has also been reported by others and may be related to the difference in viscosity between the *in vitro* calibration solutions and the intracellular environment (17, 18). The adverse effects that SBFI has on contractility clearly require that, following dye-loading, measurements of cellular viability must precede experimentation.

Inhibition of the Na^+ pump, by either application of ouabain or removal of $[K^+]_o$, produced the expected rise in $[Na^+]_i$, which increased an average of 10 mM in 5 min (Table 1). From this rate of increase, and given an average surface area of 5.88×10^{-5} cm^2 (19) and an average volume of 5.6 pl for these smooth muscles, we calculate that the passive sodium permeability of these cells was 3.17 pmol/cm^2 per sec. This is the passive Na^+ influx through the membrane and is therefore presumably the basal influx that must be balanced by the Na^+ pump in a resting cell. Since the basal turnover rate of the Na^+ pump in this tissue is 10,000 per min (1) there must be, on average, about 40 pumps per μm^2. This is similar to the 83–100 pumps per μm^2 predicted from [^{3}H]ouabain binding in the guinea pig taenia coli (20, 21). However, as passive Na^+ permeability is known to decrease as $[Na^+]_i$ rises (22), the Na^+ permeability that we have calculated is therefore the average permeability during the time that the Na^+ pump was inoperative. In spite of some uncertainties in our estimate of passive influx of Na^+, our measured value is close to the passive Na^+ permeability calculated in this cell type from radioisotope flux analysis, 1.95 pmol/cm^2 per sec (6).

In these single smooth muscle cells ISO produced a rapid and large decrease in $[Na^+]_i$, which averaged 7 mM in 3 sec (Table 1), and this requires that the stimulated rate of Na^+ efflux was 236 pmol/cm^2 per sec.‡ The total $[Na^+]_{cell}$ (free plus bound) associated with these cells measured with flame photometry was 65.5 mM, and radioisotope flux analysis had predicted that stimulation with ISO would reduce the total $[Na^+]_{cell}$ by 8%. In this study ISO decreased the free $[Na^+]_i$ 50–55%, but this corresponds to a decrease of 10.7% in total $[Na^+]_{cell}$ close to that predicted from radioisotope flux analysis. Since the Na^+ efflux rate increased from 3.17 pmol/cm^2 per sec to 239 pmol/cm^2 per sec, the rate of Na^+ efflux was transiently accelerated 75.4-fold. This is again very close to the increase in Na^+ pump activity predicted from radioisotope flux analysis (1). In the flux experiments there was a 7.6-fold increase in Na^+ pump activity recorded 30 sec after application of ISO, the first time point that was technically possible to measure. The present results, having a higher temporal resolution, indicate that the response to ISO is complete in 3 sec, and therefore the stimulation of the Na^+ pump predicted from flux analysis in 3 sec must be almost 76-fold. The stimulated increase in pump activity may represent an increase in affinity and/or V_{max} of preexisting pumps or, alternatively, may result from pump recruitment. Since a decrease in $[Na^+]_i$ in response to ISO was completely abolished by removal of $[K^+]_o$, this stimulated efflux must have been mediated entirely through the Na^+ pump. While the apparent acceleration of the Na^+/K^+ pump is considerably larger than observed in most other systems (37), even larger (>1000) increases have been noted in the electric organ (38). The mechanism responsible for this very large acceleration of the pump remains to be determined. The speed with which the Na^+ pump responds to β-adrenergic stimulation and decreases $[Na^+]_i$ is similar to the speed with which there are significant increases in cAMP, cAMP-dependent protein kinase activity, and a decrease in contractility (1). The speed of the effect of ISO on Na^+ is thus consistent with the hypothesis that an increase in Na^+ extrusion mediated by the Na^+/K^+ pump is responsible for accelerating Ca^{2+} removal from the cell, which, as we know, decreases 2.5 sec after application of ISO (3). In addition to a decrease in $[Na^+]_i$, it is also expected that acceleration of the Na^+ pump would, if it did not cause other ion movements, lead to a very large hyperpolarization.§ Although there is a hyperpolarization that is observed in response to ISO (23), it is relatively small (11 mV) and is principally due to a cAMP-mediated increase in K^+ conductance (24). The absence of a large hyperpolar-

†The basal sodium influx, and therefore efflux in this tissue, is 3.2 pmol/cm^2 per sec (see *Discussion*); with a cell surface area of 5.88×10^{-5} cm^2 per cell the change in $[Na^+]_i$ would be 1.9×10^{-4} pmol/sec, or, in 3 sec, 5.7×10^{-4} pmol. The cell volume is 5.6 pl and therefore the change in $[Na^+]_i$ expected if passive sodium influx was simply turned off would be 0.1 mM.

‡This calculation assumes that, as in other cells, there is no buffering of $[Na^+]_i$, and a change in free $[Na^+]_i$ represents a change in total $[Na^+]_i$ (17).

§To decrease $[Na^+]_i$ 7 mM in 3 sec from a volume of 5.6 pl, the Na^+ pump must move 7.89×10^9 Na^+ ions per sec per cell. With a coupling ratio of 3:2, this represents a net positive efflux of 2.63×10^9 ions per sec, or a current of 421 pA. Across an input resistance of 1.6 GΩ (A. Guerrero, personal communication) this would produce a potential difference of 0.7 V.

ization due to the large acceleration of the Na^+/K^+ pump is most likely accounted for by Cl^- movement out of the smooth muscle cell with Na^+ during pump acceleration, thereby making this process largely electroneutral. Although we have no direct information regarding Cl^- movements during the action of ISO in these cells, in other smooth muscle a significant decrease in Cl^- content has been detected by electron probe microanalysis following the action of ISO (2).

How is activation of the Na^+ pump and a decrease in $[Na^+]_i$ linked to ISO's negative inotropic effects? We know that these are not simply coincidental effects since roughly half of the negative inotropic effect of ISO is inhibited by ouabain and therefore linked to activation of the Na^+ pump. We believe that activation of the Na^+ pump and a decrease in $[Na^+]_i$ shifts the Na^+/Ca^{2+} exchanger into Ca^{2+} extrusion mode, reducing cytoplasmic $[Ca^{2+}]$ and intracellular Ca^{2+} stores and therefore the magnitude of subsequent contractions. Given that $[Na^+]_i$ is $\approx$13 mM, and $[Ca^{2+}]_i$ is $\approx$120 nM (A. Guerrero, personal communication), the reversal potential of the Na^+/Ca^{2+} exchanger is -63 mV. This is close to the resting membrane potential of these cells, -56 mV (23), and therefore at rest the Na^+/Ca^{2+} exchanger would be relatively silent. After β-adrenergic activation, however, $[Na^+]_i$ falls to $\approx$5 mM, which shifts the reversal potential of the Na^+/Ca^{2+} exchanger to +10 mV, while the membrane hyperpolarizes 11 mV to -67 mV. The new reversal potential is positive to the resting potential and therefore the Na^+/Ca^{2+} exchanger would be expected to adopt a Ca^{2+} extrusion mode and expel Ca^{2+} from the cell. A Ca^{2+} extrusion that can be blocked with either ouabain or removal of $[Na^+]_o$ has been observed in these cells, supporting this hypothesis (25).

Since the effect of ISO could be mimicked by forskolin and 8-Br-cAMP, it is most likely that Na^+ pump stimulation is mediated via adenylate cyclase activation, an increase in cAMP, and activation of cAMP-dependent protein kinase, increases that have all been observed within a few seconds after the application of ISO to these cells. Stimulation of Na^+/K^+ pump activity has been reported in other cell systems either in response to agonists believed to act by increasing cAMP-dependent protein kinase activity (26, 27) or in response to microinjection of the active kinase itself (28). We do not know whether the effects that we have observed are a result of direct phosphorylation of the Na^+ pump by a cAMP-dependent protein kinase or whether there are other proteins that may be substrates for the kinase, which directly or indirectly affect the activity of the Na^+ pump. There is precedent for both possibilities. *In vitro* and *in vivo* the α, but not the β, subunit of the pump is a substrate for protein kinase A (PKA) (29–32), and for protein kinase C (PKC) (31). Phosphorylation of the α chain by either protein kinase was always inhibitory. In intact cells Na^+ pump inhibition has been demonstrated in response to agents believed to act through PKC (33–36). The observed stimulatory effect of PKA on the Na^+/K^+ pump may therefore reflect the presence of additional factors that render the response of the pump to phosphorylation by PKA stimulatory or, alternatively, may reflect phosphorylation of other proteins by PKA, which have an overiding stimulatory effect on the Na^+/K^+ pump. Although the molecular details remain to be elucidated, the current data clearly indicate that stimulation of cAMP-dependent protein kinase causes a profound stimulation of the Na^+ pump in intact smooth muscle cells.

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