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3-Isobutyl-1-methylxanthine Inhibits Sustained Calcium Current Independently of Cyclic AMP in Neuronal and Endocrine Cells

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SUMMARY

The effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) on the voltage-dependent calcium current was studied in the clonal pituitary cell line GH_3 by whole-cell patch-clamp techniques. It was found that IBMX reversibly inhibited the sustained calcium current (K_i, 1.25 mM), whereas there was no effect on the transient current. IBMX increased the inactivation rate of the sustained current without altering the voltage of activation. Vasoactive intestinal peptide, an agent known to increase cAMP, was without effect on the calcium current. The effect of IBMX was not altered by pretreating the cells with pertussis toxin or by including either cAMP or protein kinase inhibitor in the intracellular solution. The order of potency for several xanthine derivatives was IBMX > theophylline > caffeine > xanthine. The effect of IBMX on calcium current was also observed in three additional neuronal and endocrine cell lines (PC12, SY5Y, and RINm5f). These results indicate that IBMX inhibits sustained voltage-dependent calcium current by a mechanism independent of alterations in cAMP levels.

It is well documented that increases in cAMP result in a large increase in the amplitude of the L-type calcium current expressed in cardiac cells (1–4). The effects of cAMP on calcium currents in other preparations remain more controversial. For example, in some neuronal preparations cAMP has been shown to weakly increase calcium currents (5–8), whereas in others there is no effect of cAMP on calcium currents (8, 9). These conflicting observations on the actions of cAMP on calcium currents also extend to endocrine and neuroendocrine cells, where both increases (10, 11) and no effect (12, 13) have been observed.

The purpose of the present study was to examine in more detail the effects of cAMP-increasing agents on the voltage-dependent calcium current expressed in the clonal pituitary cell line GH_3. It was previously reported that inclusion of cAMP in standard whole-cell patch-clamp recordings from GH_3 cells caused no change in the calcium current (12). On the other hand, it was reported that 8-bromo-cAMP slowed the calcium-dependent inactivation of the dihydropyridine-sensitive calcium current (14). Furthermore, addition of activated protein kinase A restored calcium channel activity that had previously run down (10). Finally, VIP, an agent known to increase cAMP in GH_3 cells (15), was reported to increase both the sustained and transient currents expressed in these cells (16), although whether these effects were mediated by cAMP was not determined.

Some of these conflicting results may be due to the loss of key intracellular factors in the standard whole-cell patch-clamp configuration. We avoided this potential artifact in the present study by using the perforated-patch configuration (17). To ensure that cAMP levels were elevated we included IBMX, an agent that prevents the breakdown of cAMP by inhibiting phosphodiesterases (18). It was found that a mixture of VIP and IBMX inhibited calcium currents in GH_3 cells, that the inhibition was due to the presence of IBMX, and that this inhibition was probably not due to the effects of IBMX on cAMP levels. Finally, it was found that this inhibition of calcium current by IBMX was not unique to GH_3 cells and was also found in several other endocrine and neuronal cell types.

Materials and Methods

Cell culture. GH_3 cells, a pituitary cell line that secretes both prolactin and growth hormone (19), were obtained from the American Type Culture Collection (Rockville, MD). GH_3 cells were grown in Ham's F-10 medium supplemented with 15% horse serum and 3% fetal calf serum.

PC12 cells, a rat pheochromocytoma cell line (20), were obtained from Dr. John Aletta (Department of Pharmacology, School of Medicine, State University of New York at Buffalo). PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum.

SY5Y cells, a human neuroblastoma cell line (21), were obtained from Dr. Stanley Halvorsen (Department of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo).

ABBREVIATIONS: VIP, vasoactive intestinal peptide; IBMX, 3-isobutyl-1-methylxanthine; PKI, protein kinase inhibitor; DMSO, dimethylsulfoxide; PTX, pertussis toxin; TTX, tetrodotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

SY5Y cells were grown in minimum essential medium/Ham's F-12 medium supplemented with 15% fetal calf serum.

RINm5f cells, a rat insulinoma (22), were obtained from Dr. Suzanne Laychock (Department of Pharmacology, School of Medicine, State University of New York at Buffalo). RINm5f cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

All cells were grown in a humidified incubator at 36° in an atmosphere of 95% air/5% CO_2. For electrophysiological experiments GH_3 cells were subcultured onto untreated glass coverslips 3–10 days before use. All other cells were subcultured onto poly-L-lysine-treated coverslips (25 μg/0.5 ml of H_2O) and used within 24 hr.

Electrophysiology. Standard whole-cell patch-clamp methods (23) and the perforated-patch variation (17) were used. Cells grown on glass coverslips were placed in a Plexiglass perfusion chamber (0.5-ml volume) mounted on an inverted phase-contrast microscope. Agents were applied to the cells by bath exchange (perfusion rate, 2 ml/min). A Ag/AgCl plug was used as a ground electrode in the effluent from the bath. Experiments were done at room temperature.

Patch electrodes (resistance, 2–6 MΩ) were pulled from TW150–6 glass (World Precision Instruments, Inc., Sarasota, FL). The perforated-patch configuration was achieved by back-filling the patch electrode with buffer containing 10 μl of nystatin stock solution/3 ml of pipet buffer. Nystatin stock solution (50 mg/ml of DMSO) was made fresh daily. In standard whole-cell recordings access resistances were typically <10 MΩ, whereas in the perforated-patch configuration access resistances were typically 30–50 MΩ.

Current recordings were made with the use of a List EPC-7 patch-clamp amplifier (Medical Systems Corp., Greenvale, NY). After a whole-cell configuration was achieved the experimental parameters were controlled by a computer equipped with Basic Fastlab software (Indec Systems, Sunnyvale, CA). Typically, cells were held at −85 mV and pulsed to the desired voltage for 150–200 msec once every 5 or 10 sec. Current signals were filtered at 3 kHz and data were sampled at 1–5 kHz. Current amplitudes were corrected for leak current. Leak currents were estimated by the averaged current response to four 30-mV hyperpolarizing voltage pulses from a holding potential of −60 mV.

Solutions. Calcium currents were isolated by blocking other current components present. Potassium currents were blocked by using Cs^+-containing pipet solutions and sodium currents were blocked by the addition of 0.5 μM TTX in the bath.

The standard bath solution contained 10 mM $CaCl_2$, 130 mM NaCl, 6 mM glucose, and 10 mM HEPES, with the pH adjusted to 7.4 with NaOH. In some experiments bath $CaCl_2$ was raised to 25 mM (with NaCl reduced to 106 mM) or bath $CaCl_2$ was replaced by $BaCl_2$. The effects seen with IBMX were identical in all bath solutions.

For perforated-patch recordings the pipet solution contained 80 mM CsCl, 30 mM Cs_2SO_4, 3 mM $MgCl_2$, and 10 mM HEPES, with the pH adjusted to 7.4 with NaOH. For standard whole-cell recordings the pipet solution contained 80 mM CsCl, 30 mM Cs_2SO_4, 4 mM $MgCl_2$, 10 mM EGTA (as the sodium salt), 2.5 mM ATP (as the sodium salt), and 10 mM HEPES, with the pH adjusted to 7.4 with NaOH. Other compounds were added to the standard whole-cell pipet solution as indicated in the text.

Data analysis and statistics. The voltage-dependent calcium current in GH_3 cells consists of transient and sustained components (24, 25). In this communication calcium current responses were analyzed as peak current (maximum inward current value), sustained current (current just before termination of the depolarizing voltage pulse), and transient current (peak current minus sustained current).

To fit data to specific equations (for exponential decays or calculation of a K_i for IBMX), the TKSolverPlus software package (Universal Technical Systems, Inc., Rockford, IL) was used. When the effect of a compound was compared with control a paired t test was used. When multiple samples of data were compared, an analysis of variance was used. For comparisons of values within multiple samples, a Newman-Keuls multiple range test was used.

Materials. All cell culture reagents were obtained from GIBCO (Grand Island, NY). All buffer salts were obtained from Sigma Chemical Co. (St. Louis, MO). The PKI (Walsh inhibitor) used was Sigma P-5015. IBMX, caffeine, theophylline, HEPES, EGTA, cAMP, ATP, PTX, TTX, DMSO, and nystatin were obtained from Sigma. VIP was obtained from Bachem (Torrance, CA).

Results

When cells were held at −80 or −85 mV and then pulsed to +10 mV, the application of 100 nM VIP was found to have little effect (Figs. 1 and 2). The amplitudes (mean ± standard error) of the peak, sustained, and transient currents were 99 ± 4%, 96 ± 4%, and 105 ± 6% of control, respectively, 1–2 min after application of VIP (n = 8, all perforated patch recordings). It was previously reported that VIP shifted the voltage of activation of both sustained and transient calcium currents to more hyperpolarized potentials (16), so the effect of VIP on the current-voltage relationship of the calcium current was examined (Fig. 2). Again, no effect of VIP was observed in our recordings.

However, the addition of 1 mM IBMX to cells treated with VIP caused a rapid decrease of the sustained current to 50–60% of control (Fig. 1). In all cases the effects of IBMX were at least partially reversible, with some cells showing complete recovery. Additional experiments demonstrated that the drop in sustained calcium current caused by the mixture of VIP and IBMX could be produced by IBMX alone (Fig. 3). This effect of IBMX was not due to the DMSO contained in the IBMX stock solution (Fig. 3).

In all cases IBMX caused a drop in both peak and sustained currents (Figs. 1 and 3). However, in the calculation of the transient current it was found that the effect of IBMX ranged

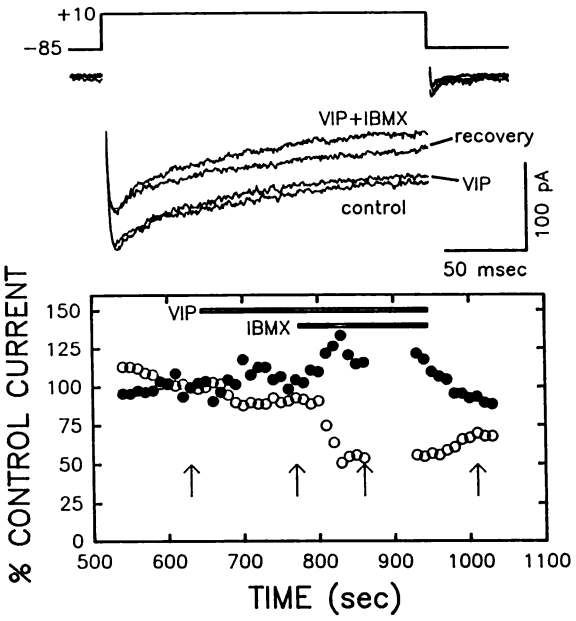

Fig. 1. Effect of VIP (100 nM) and IBMX (1 mM) on the calcium current in a GH_3 cell, recorded in the perforated-patch configuration with 10 mM bath Ca^{2+}. *Top trace* in this and subsequent figures, voltage protocols. *Current traces* shown are from recordings indicated by *arrows on the graph*. In this and most subsequent recordings the first 1.5 msec after a voltage change has been omitted for clarity. Control current is that obtained at the *first arrow*. *Bars on the graph*, times when bath inflow was changed to the indicated solutions. Time is from formation of the gigaseal. ○, Sustained current levels; ●, transient current levels.

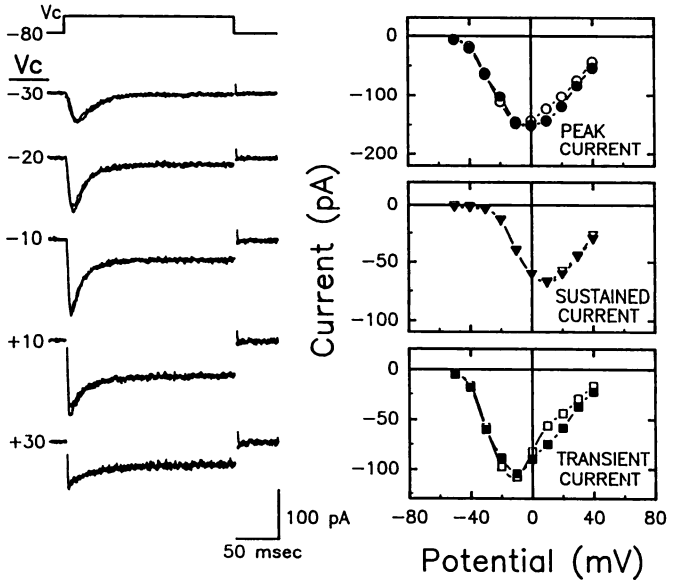

Fig. 2. Effect of VIP (100 nM) on the voltage-current relationship of the peak, sustained, and transient calcium currents recorded in the perforated-patch configuration with 10 mM bath Ca^{2+}. *Left*, *traces* in response to the command voltage (*Vc*) indicated to the *left* of each set. *Right*, *open symbols*, control values; *closed symbols*, values obtained in the presence of VIP. Similar results were obtained in two additional cells.

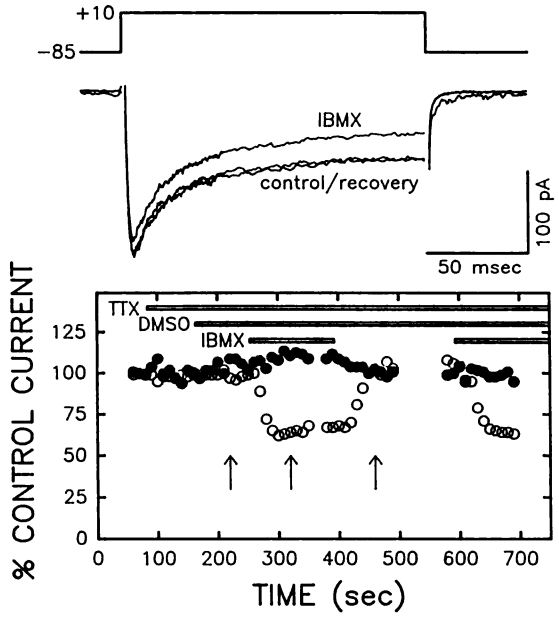

Fig. 3. Effect of TTX (0.5 μM), DMSO (0.1%, v/v), and IBMX (1 mM) on the calcium current in a GH_3 cell, recorded in the standard whole-cell configuration with 25 mM bath Ca^{2+}. *Current traces* shown are from recordings indicated by *arrows on the graph*. Control current is that obtained after TTX was washed onto the cell. *Bars on the graph*, times when bath inflow was changed to the indicated solutions. ○, Sustained current levels; ●, transient current levels.

from no effect (Fig. 3) to a rather large apparent increase (>20%) (Fig. 1). It was found that, the smaller the relative magnitude of the transient current, the greater the tendency for IBMX to increase the estimated size of this component (data not shown).

At least part of this increase, if not all, is due to the relatively crude separation of the transient current from the sustained

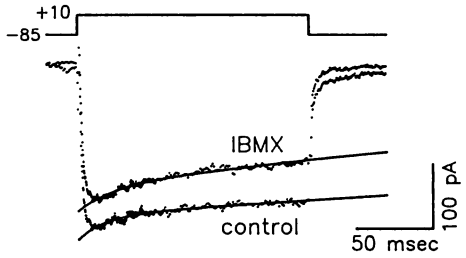

Fig. 4. Double-exponential fits to the inactivating portion of the calcium current in GH_3 cells, recorded in the standard whole-cell configuration with 25 mM bath Ca^{2+}. Average values from these fits and two additional fits for each condition were as follows: control: fast rate, 58.6 ± 9.0 sec^{-1}; amplitude of fast component, −26.8 ± 6.3 pA; slow rate, 1.84 ± 0.07 sec^{-1}; amplitude of slow component, −107 ± 1 pA; IBMX: fast rate, 63.0 ± 8.9 sec^{-1}; amplitude of fast component, −35.3 ± 0.5 pA; slow rate, 3.52 ± 0.32 sec^{-1}; amplitude of slow component, −85.9 ± 4.3 pA. Changes caused by IBMX were reversible. These results and results from analyses of three additional cells are summarized in the text.

current. Exponential fits to the inactivating portion of the calcium current revealed two inactivation rates (Fig. 4). Thus, part of the calculated transient current was contaminated by the component of sustained current that inactivated over the period of the voltage pulse. Furthermore, it was found that application of IBMX caused a statistically significant increase in the inactivation rate of the sustained current component (Fig. 4) (mean ± standard error of the slow inactivation rate was 1.31 ± 0.24 sec^{-1} before and 2.33 ± 0.44 sec^{-1} after 1 mM IBMX; $n = 4$; $p < 0.002$, paired t test). This increase in the slow inactivation rate would tend to cause an overestimation of the fast transient current component by the crude separation methods used in this study, especially when the transient current was small relative to the sustained component.

On the other hand, IBMX caused no change in the fast inactivation rate (53.5 ± 9.2 sec^{-1} before and 53.6 ± 7.1 sec^{-1} after IBMX; mean ± standard error; $n = 4$). Although, as determined from the amplitudes of the exponential fits, the fast component did have a tendency to increase after IBMX (47 ± 25%; mean ± standard error; $n = 4$), this increase was not statistically significant. In contrast, the decrease in the amplitude of the slow inactivating component was found to be statistically significant (−23 ± 5%; mean ± standard error; $n = 4$; $p < 0.05$, paired t test), consistent with the observed decrease when sustained current was measured at the end of the voltage pulse.

Examination of the effects of 1 mM IBMX on the current-voltage relationship of the calcium current supports the contention that the major effect of IBMX is on the sustained calcium current (Fig. 5). At weak depolarizing voltages (−29 and −19 mV), IBMX had little effect on the calcium current (Fig. 5). At these voltages the current is dominated by the T-type current. At stronger depolarizing voltages (−10, +10, and +29 mV) the effects of IBMX were more apparent (Fig. 5). At these voltages the sustained or L-type current becomes a significant portion of the total current. These conclusions are illustrated in the graphs in Fig. 5, which show little effect of IBMX on the transient current but a large effect of IBMX on the sustained component. IBMX did not cause any apparent shift in the activation voltage of the sustained current (Fig. 5, *middle graph*).

Because of the apparent specificity of IBMX effects on the sustained component of current, in all subsequent analyses the

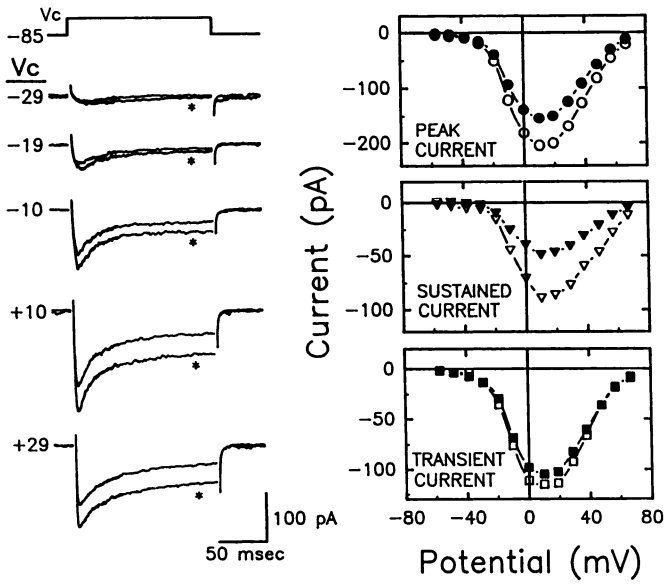

Fig. 5. Effect of IBMX (1 mM) on the voltage-current relationship of the peak, sustained, and transient calcium currents recorded in the standard whole-cell configuration with 25 mM bath Ca^{2+}. *Left*, *traces* in response to the command voltage (*Vc*) indicated to the *left* of each set. *, Control current trace. *Right*, *open symbols*, control values; *closed symbols*, values obtained in the presence of IBMX.

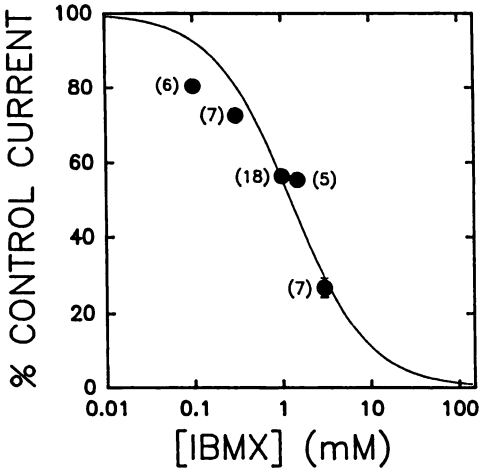

Fig. 6. Dose-response relationship for the effects of IBMX on the sustained calcium currents. Conditions were the standard whole-cell configuration with 10 mM Ba^{2+} in the bath. *Numbers next to each symbol*, number of independent determinations for the value. Values reported are mean ± standard error (for most values the standard error is smaller than the symbol). The *line* drawn is the best fit to the following equation: current = 100/(1 + [IBMX]/K_i). The value for K_i from the fit is 1.25 mM.

effects of IBMX are reported only for the sustained component of current. Furthermore, IBMX had the same effect on the calcium current whether the perforated-patch configuration (Fig. 1) or the standard whole-cell configuration (Fig. 3) was used. Thus, results from these two configurations are combined when other conditions were similar. Finally, it was found that 1 mM IBMX had the same effect regardless of whether Ca^{2+} was the current carrier (41 ± 1% inhibition; n = 11) or Ba^{2+} was the current carrier (44 ± 1% inhibition; n = 18).

The apparent K_i for IBMX was found to be 1.25 mM (Fig. 6). The fit to the data was not particularly good at low concentrations of IBMX. This is probably due to the contamination of the data with a slight degree of calcium current rundown. As a relative proportion of inhibition, rundown would be a much more significant effect at low concentrations of IBMX.

In the examination of the effects of related xanthines, IBMX was found to be the most potent (Fig. 7). It required 10 mM theophylline and 20 mM caffeine to achieve the same degree of inhibition as that produced by 1 mM IBMX. Xanthine, at 5 mM (the highest concentration tested), had no effect.

Two different strategies were applied to determine whether the effects of IBMX were mediated by changes in cAMP. The first involved adding 2 mM cAMP to the pipet solution in the standard whole-cell configuration. This concentration would saturate protein kinase A (26). Furthermore, given the large reserve of cAMP in the pipet, the ability of phosphodiesterases to decrease cAMP should be overwhelmed. Under these conditions, if the decrease in calcium current caused by IBMX was due to an increase in cAMP, it should not be observed. However, inclusion of 2 mM cAMP in the pipet had no influence on the effect of IBMX (Fig. 8). Similar results were obtained when the pipet was loaded with 10 mM cAMP (data not shown).

The second strategy was to include PKI in the pipet (0.5 mg/ml). The effects of IBMX were not different when PKI was included in the pipet (Fig. 8). These two results indicate that IBMX does not inhibit sustained calcium currents by increasing cAMP levels.

It has been shown by others that sustained calcium currents can be directly inhibited by PTX-sensitive G proteins (9, 13).

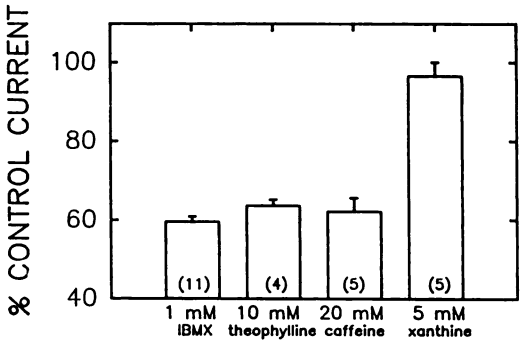

Fig. 7. Effects of various xanthine analogs on the sustained calcium current in GH_3 cells. Conditions were the standard whole-cell configuration with 10 mM Ca^{2+} in the bath. *Numbers in parentheses*, number of independent determinations. *Bars*, means ± standard errors.

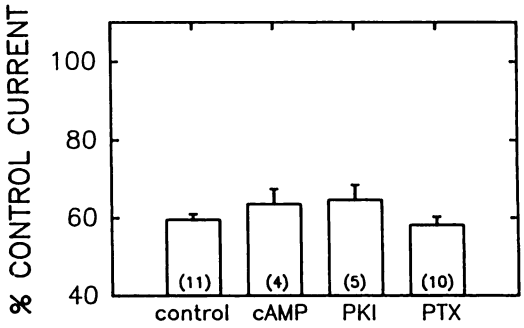

Fig. 8. Effects of various agents on the ability of IBMX (1 mM) to inhibit sustained calcium current in GH_3 cells. Conditions for control, cAMP (2 mM in pipet solution), and PKI (0.5 mg/ml in pipet solution) were the standard whole-cell configuration with 10 mM bath Ca^{2+}. The conditions for PTX-pretreated cells (0.1 μg/ml overnight) were a mixture of standard whole-cell and perforated-patch configurations with either 10 mM Ba^{2+} or 10 mM Ca^{2+} in the bath. *Numbers in parentheses*, number of independent observations. *Bars*, means ± standard errors.

To eliminate the possibility that IBMX was also working via a PTX-sensitive G protein, we examined the effects of IBMX on cells treated overnight with 0.1 μg/ml PTX. We found that PTX treatment had no effect on the ability of IBMX to inhibit the sustained calcium current (Fig. 8).

To determine whether this effect of IBMX was unique to GH_3 cells or could also be found in other preparations, we studied the effects of 1 mM IBMX on the calcium current expressed by the rat insulinoma cell line RINm5f, the rat pheochromocytoma cell line PC12, and the human neuroblastoma cell line SY5Y. We found that IBMX also decreased the sustained calcium current in all these cells (Fig. 9). However, IBMX was most potent in GH_3 cells, followed by SY5Y, PC12, and RINm5f cells. The differences in degree of inhibition were found to be statistically significant (analysis of variance, $p < 0.001$).

Discussion

The results from these experiments demonstrate that IBMX inhibits the sustained calcium current in endocrine pituitary (GH_3), endocrine pancreatic (RINm5f), neuroendocrine (PC12), and neuronal (SY5Y) cell lines. It is unlikely that this effect is mediated by increases in cAMP, because saturation of the intracellular solution with cAMP or addition of protein kinase A inhibitor to the intracellular solution had no effect on the ability of IBMX to inhibit sustained calcium current. Furthermore, this effect is not mediated by a PTX-sensitive G protein, because pretreatment of the cells with PTX had no effect on the ability of IBMX to inhibit the sustained calcium current. These results suggest that IBMX acts either directly on the calcium channel itself or on some closely related regulatory protein (27).

The K_i for the effect of IBMX on calcium currents in GH_3 cells was found to be 1.25 mM. This is 1 order of magnitude higher than the K_i reported for IBMX inhibition of phosphodiesterase activity (18). However, to achieve 100% inhibition of phosphodiesterase activity, concentrations as high as 500 μM are required. Our results suggest that careful consideration should be given to the interpretation of experiments in which such high concentrations of IBMX are used to inhibit phosphodiesterases. Likewise, although caffeine is much less potent than IBMX in the inhibition of calcium currents, our results also suggest caution in the interpretation of experimental results in which concentrations of caffeine greater than 10 mM are used.

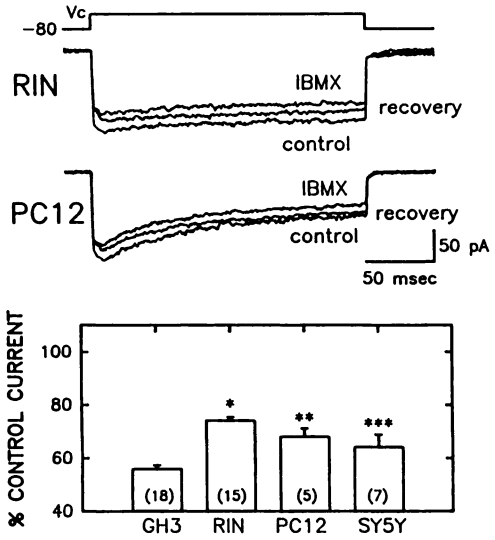

Fig. 9. Comparison of effect of IBMX (1 mM) on sustained calcium currents in GH_3, RINm5f (*RIN*), PC12, and SY5Y cells. Conditions were the standard whole-cell configuration with 10 mM Ba^{2+} as the current carrier. Some values for RIN cells were obtained with 10 mM bath Ca^{2+}. *Upper*, *sample traces* from RIN and PC12 cells. *Numbers in parentheses*, number of independent observations. *Bars*, means ± standard errors. *, Significantly different from SY5Y ($p < 0.01$) and GH_3 ($p < 0.001$); **, significantly different from GH_3 ($p < 0.01$); ***, significantly different from GH_3 ($p < 0.05$).

Phosphodiesterase inhibitors related to trapidil have been reported to inhibit calcium currents in neuroblastoma × glioma cells (28) and in cardiac ventricular cells (29). These trapidil derivatives were shown to increase the slow inactivation rate without altering the voltage dependence of activation (29), a result consistent with the effects we observed with IBMX. Whether IBMX is exerting its effects via the same mechanism as these trapidil derivatives remains to be determined.

In apparent contradiction to our results, it has been reported that high concentrations of IBMX have a profound effect in increasing the L-type calcium current in ventricular cells (1, 4). This contradiction with the present data may be explained by the fact that agents that increase cAMP increase the L-type calcium current in cardiac cells by 3–4-fold (2, 3), whereas the regulation in neuronal and endocrine preparations is modest, if at all present (5–13). Thus, the possibility that IBMX decreases the calcium current by 20–50% in cardiac cells would be overridden by the large increase in the current caused by increases in cAMP levels.

On the other hand, it could be that IBMX has no ability to inhibit the cardiac L-type channel. Recent cloning data demonstrate that the structure of the α1 subunit of the cardiac L-type channel is slightly different from that of the α1 subunit of the L-type channel found in neuronal/endocrine preparations (30). If IBMX acts directly on the α1 subunit, then this difference in structure may underlie the lack of inhibition of the cardiac L-type channel observed by others. Small differences in structure or differences in the expression of proteins that regulate the calcium channel (27) may also underlie the slightly different abilities of IBMX to inhibit the sustained current in the different neuronal and endocrine preparations studied in this report.

We were unable to confirm the previously reported effects of VIP in increasing the transient and sustained calcium currents in GH_3 cells (16). The reasons for this are not apparent, although Hedlund *et al.* (16) used 1 μM VIP, compared with 100 nM in our study. It is unlikely that our failure to observe these effects of VIP was due to overbuffering of intracellular Ca^{2+}, an effect reported to block VIP actions. In our recordings we used the perforated-patch configuration, which should minimize disturbances to the intracellular milieu.

However, our results support the finding of Rosenthal *et al.* (12) that inclusion of cAMP in the pipet has little effect on calcium currents in GH_3 cells. Our results do not contradict those of Kalman *et al.* (14). Those authors found that treatment with 8-bromo-cAMP slowed Ca^{2+}-induced inactivation of the L-type current. In our standard whole-cell recordings Ca^{2+} was buffered so heavily (10 mM EGTA) that Ca^{2+}-induced inactivation was unlikely to occur.

In conclusion, we found that IBMX inhibited the sustained

calcium current in neuronal and endocrine cells. This effect of IBMX was insensitive to manipulations that should alter the activity of protein kinase A, suggesting that the effect was mediated directly via the calcium channel protein or a closely related protein. Further study of the mechanism of this inhibition could provide more insights into the differences between the L-type channel expressed in cardiac cells versus neuronal and endocrine preparations. Finally, this action of IBMX should be considered in the interpretation of experiments in which high concentrations of IBMX are used to inhibit phosphodiesterases.

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