Differential Tyrosine Phosphorylation of Plasma Membrane Ca^{2+}-ATPase and Regulation of Calcium Pump Activity by Carbachol and Bradykinin*

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We investigated the effects of thapsigargin (TG), bradykinin (BK), and carbachol (CCh) on Ca^{2+} entry via endogenous channels in human embryonic kidney BKR21 cells. After depletion of Ca^{2+} stores by either TG, BK, or CCh, the addition of Ca^{2+} gave a much larger rise in Ca^{2+} levels in CCh-treated and TG-treated cells than in cells treated with BK. However, in experiments performed with Ba^{2+}, a cation not pumped by Ca^{2+}-ATPases, only a modest difference between CCh and BK-stimulated Ba^{2+} entry levels was observed, suggesting that the large difference in the Ca^{2+} response is mediated by a differential regulation of Ca^{2+} pump activity by CCh and BK. This hypothesis is supported by the finding that when Ca^{2+} is removed during the stable, CCh-induced Ca^{2+} plateau phase, the decline of cytosolic Ca^{2+} is much faster in the absence of CCh than in its presence. In addition, if Ca^{2+} is released from a caged Ca^{2+} compound after a UV pulse, the resulting Ca^{2+} peak is much larger in the presence of CCh than in its absence. Thus, the large increase in Ca^{2+} levels observed with CCh results from both the activation of Ca^{2+} entry pathways and the inhibition of Ca^{2+} pump activity. In contrast, BK has the opposite effect on Ca^{2+} pump activity. If Ca^{2+} is released from a caged Ca^{2+} compound, the resulting Ca^{2+} peak is much smaller in the presence of BK than in its absence. An investigation of tyrosine phosphorylation levels of the plasma membrane Ca^{2+}-ATPase (PMCA) demonstrated that CCh stimulates an increase in tyrosine phosphorylation levels, which has been reported to inhibit Ca^{2+} pump activity, whereas in contrast, BK stimulates a reduction of PMCA tyrosine phosphorylation levels. Thus, BK and CCh have a differential effect both on Ca^{2+} pump activity and on tyrosine phosphorylation levels of the PMCA.

When cells are stimulated with agonists for G-protein-coupled receptors (GPCRs) coupled to G_s, the resulting Ca^{2+} response is generally biphasic in nature. The initial peak of the response is because of the rapid release of Ca^{2+} from internal stores in response to inositol 1,4,5-trisphosphate generation. This initial peak is followed by a lower but longer lasting plateau phase that results from Ca^{2+} entry via plasma membrane Ca^{2+} channels. A substantial portion of this Ca^{2+} entry is via capacitative calcium channels, also called store-operated calcium channels, as initially described by Putney (1). Since the original description of store-operated calcium channels, much work has been done to investigate what proteins mediate store-operated Ca^{2+} entry (SOCE) and how these channels are regulated. There is substantial evidence that the Drosophila Trp protein can function as a store-operated Ca^{2+} channel (2, 3). Recently a number of papers have described investigations into whether mammalian Trp homologs can also function as store-operated Ca^{2+} channels, reviewed by Minke (4). Although some of the early papers supported the hypothesis that mammalian Trp homologs mediate store-operated calcium entry (5–7), a number of other papers argued that these Trp homologs mediate other types of Ca^{2+} entry. For example, several recent papers report that overexpression of either human TrpC3 (hTrpC3) or murine TrpC6 gives a much lower level of Ca^{2+} entry in response to depletion of internal Ca^{2+} stores by thapsigargin (TG) but gives a much larger Ca^{2+} entry in response to carbachol (CCh) (8, 9). These observations have led some to the interpretation that hTrpC3 and murine TrpC6 may code for receptor-operated rather than store-operated Ca^{2+} channels.

From our previous investigations of endogenous Ca^{2+} channels in HEK-293 cells, we knew that the level of Ca^{2+} entry in response to depletion of internal Ca^{2+} stores by TG was always significantly larger than the level of Ca^{2+} entry in response to stimulating the G-protein-coupled receptor for bradykinin (BK). Because this result was in contrast to the CCh results reported for HEK-293 cells overexpressing various Trp isoforms, we were interested in determining whether there was something fundamentally different about the stimulation of Ca^{2+} entry by CCh in comparison to BK. For example, does CCh release more Ca^{2+} from internal stores than BK, thereby giving a higher store-operated Ca^{2+} entry, or does CCh stimulate a receptor-operated Ca^{2+} channel that for some reason is not activated by BK? On the other hand, perhaps the exogenously expressed hTrpC3 and murine TrpC6 channels behave differently than the channels endogenous to HEK-293 cells. To investigate these questions, we performed a detailed comparison of the effects of CCh, BK, and TG on Ca^{2+} entry via endogenous channels in HEK-293 cells, which we have previously demonstrated express mRNA coding for endogenous hTrpC1, hTrpC3, hTrpC4, and hTrpC6 channels (10). In addition, we have recently confirmed that HEK-293 cells express endogenous hTrpC1, hTrpC3, and hTrpC4 proteins (11).
**EXPERIMENTAL PROCEDURES**

**Materials**—HEK-293 cells were obtained from the Richard Miller laboratory (Northwestern University, Chicago, IL). The cDNA encoding for the hB2-BKR was obtained from Fred Hess (Merck). Fura-2 acetoxymethyl ester (Fura-2-AM), Fura-2 free acid, Fluor-3-AM, nitrophenyl-EGTA-AM (NP-EGTA-AM), and Pluronic F-127 were purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals were purchased from Sigma.

**Cell Culture**—HEK-293 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. Cells were subcultured onto 25-mm round coverslips 1 day before experiments. HEK-293 cells were stably transfected with the hB2-BKR cDNA construct using the calcium phosphate method. The cDNA for the B2 receptor was originally cloned from the lung fibroblast cell line CCD-16Lu into the eukaryotic expression vector pcDNA I-Neo (12). G418-resistant clones were selected, and 48 clones were assayed for B2-BKR expression by Fura-2 imaging. A clone (HEK-BKR21) was selected for use in all experiments because it expresses a similar number of BK binding sites to that expressed in normal human fibroblasts. These cells, however, express only endogenous TrpC channels and carbachol receptors. Clones were cultured in the presence of 400 µg/ml G418 and used for 20–30 passages.

**Calcium Imaging**—Cells were loaded with 5 µM Fura-2-AM in HEPES-buffered Hanks' balanced salt solution (HBSS) plus 1 mg/ml bovine serum albumin plus 0.025% Pluronic F127 detergent for 30 min at room temperature and incubated without Fura-2-AM in HBSS for 30 min. To monitor intracellular [Ca²⁺]i, the glass coverslips were placed in a perfusion chamber and mounted onto the stage of a Nikon inverted epifluorescence microscope. The cells were excited with alternating 340- and 380-nm light, and the emission was measured at 510 nm.

**Uncaging**—For the experiments using NP-EGTA "caged calcium," HEK-BKR21 cells were plated on coverslips 1 day before the experiment. The next morning, cells were preincubated with NP-EGTA-AM (6 µM, dissolved in loading buffer) at 37 °C for 1.5 h. Cells were then removed from the incubator, and they were loaded with fluorescent indicator Fluo-3-AM for 30 min at room temperature (5 µM, added into the NG-EGTA-AM loading buffer). Cells were then incubated with HEPES-buffered HBSS for a 30-min unloading period. The coverslips were mounted at the bottom of a perfusion chamber that was placed on the stage of a Nikon Diaphot inverted epifluorescence microscope. An InCyt Im1 UN uncoupling module, and software (Intracellular Imaging Inc., Cincinnati, OH) was used to uncage the Ca²⁺ from the cytosolic NP-EGTA during the experiment. The UV light for uncaging is delivered via the microscope objective. The uncaging module works with two excitation filters, the uncaging filter and a Fluoro-3 excitation filter. The uncaging signal was initiated by moving the filter wheel to the uncaging filter position (330 nm) to deliver a burst of UV light (from a 300 watt xenon lamp), and then the filter wheel was switched to the Fluor-3 excitation filter position (485 nm) for excitation of the Fluoro-3 to continue the Ca²⁺ measurement. The UV photolysis, achieved by a 1-s exposure followed by a 1-s interval, repeated 10 times, resulted in a rapid, transient increase in [Ca²⁺]i. Fluoro-3-AM fluorescence intensity was measured for each coverslip as an average for ¬400 cells. The background-corrected fluorescence intensity was reported in the figures. Control studies were carried out on cells with and without preincubation in NP-EGTA-AM, which demonstrated distinctive [Ca²⁺]i responses.

**Calcium Imaging in HEK-BKR21 Cells**

In HEK-BKR21 cells, stimulated with either BK or CCh, the intracellular Ca²⁺ concentration ([Ca²⁺]i) was monitored with Fura-2 imaging. After the establishment of a baseline, cells were stimulated with 100 nM BK (panel A) or 100 µM CCh (panel B), both saturating concentrations. After 5 min of treatment, plateau levels were measured.

**Differential Regulation of PMCA by CCh and BK**

Calcium Imaging—HEK-BKR21 cells were stimulated with 100 nM BK or 100 µM CCh, and the intracellular Ca²⁺ concentration (i[Ca²⁺]i) was monitored with Fura-2 imaging. After the establishment of a baseline, cells were stimulated with 100 nM BK (panel A) or 100 µM CCh (panel B), both saturating concentrations. After 5 min of treatment, plateau levels were measured.
strated that the presence of the caged calcium compound did not change the basal level of \([\text{Ca}^{2+}]_{i}\) or the maximal response to CCh or BK stimulation (data not shown). Also, the fact that the BK and CCh peak heights were the same before and after flash photolysis demonstrated that the repeated, brief (1 s) UV exposures did not lead to photodamage of the cells during the experimental period.

**Immunoprecipitations and Immunoblotting**—Cells were grown on 10-cm dishes to confluence. Experiments were performed as follows. Cells were incubated at 37 °C for 2 h in HBSS then incubated with 100 nM BK for 2 min or with 100 μM CCh for 2 min. Cells were lysed in modified radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 2 mM NaVO₄, 2 mM Na₃P₂O₇, 2 mM NaF). Two milligrams of total protein from each lysate were used for each immunoprecipitation reaction as well as 5 μg of anti-phosphotyrosine antibody, clone PY20 (Upstate), or 2 μg of anti-plasma membrane Ca²⁺-ATPase (anti-PMCA) antibody, clone 5F10 (Affinity Bioreagents). Lysates plus antibody were incubated overnight at 4 °C with continuous rotation, then 100 μl of protein A-Sepharose (50% solution) was added and incubated again for 1 h at room temperature with continuous rotation. Then the Sepharose was washed with cold radioimmune precipitation assay buffer 4 times, and proteins were eluted from Sepharose by adding 2× Laemmlí buffer (plus 100 mM dithiothreitol) and heated for 5 min at 95 °C, and samples were loaded on 7.5% SDS-PAGE. Electrophoresis was performed, and then proteins were electrotransferred onto polyvinylidene difluoride Immobilon membranes (Millipore). The membranes were blocked with 5% milk solution in Tris-buffered saline, 0.1% Tween 20 for 1 h and were then incubated with primary antibodies raised against PMCA (clone 5F10) overnight at room temperature. The antibodies were diluted 1:3000 in the blocking solution. Membranes were washed 4 × 15 min with Tris-buffered saline, 0.1% Tween 20, incubated for 30 min at room temperature with secondary anti-mouse antibody (1:10000 in Tris-buffered saline, 0.1% Tween 20), washed under the same conditions, and developed with SuperSignal chemiluminescent substrate (Pierce) at a suitable time so as not to saturate the film. The films were digitized on a flatbed scanner, and the relative spot intensities were determined in Photoshop 6.0. The bands were outlined, and a measure of the average intensity and the pixel number was used as a measure of the integrated spot intensity. Because the basal integrated intensity can be influenced by a number of factors that might vary from experiment to experiment, data were expressed for each experiment in terms of a ratio of the stimulated to the basal value for comparisons to other experiments.

**RESULTS**

Although we had never compared them in the same experiment, our previous experience using BK and TG to stimulate...
Ca\(^{2+}\) entry in HEK-BKR21 cells indicated that depleting Ca\(^{2+}\) stores with TG would give a more robust Ca\(^{2+}\) entry than activating the GPCR for BK. This result was in stark contrast to those reported in HEK-293 cells overexpressing hTrpC3, where stimulation of the CCh GPCR gave a more robust Ca\(^{2+}\) entry than did stimulation of cells with TG (9). Thus, we were interested in determining whether there is a fundamental difference between the way BK and CCh stimulate Ca\(^{2+}\) entry in HEK-BKR21 cells. We began our study by comparing the Ca\(^{2+}\) response initiated by BK and CCh in HEK-BKR21 cells that were expressing only endogenous calcium channels. We incubated cells in HBSS and added agonist to determine whether the plateau phase differed in cells stimulated with either BK or CCh. As seen in Fig. 1, CCh stimulated a significantly higher plateau phase of [Ca\(^{2+}\)] than did BK. The mean value for the CCh-stimulated plateau was 138.4 ± 13.6 (n = 5) with a basal level of 53.6 ± 8.5 (n = 5) compared with the CCh-stimulated plateau of 71.5 ± 8.6 (n = 4) with a basal level of 48.8 ± 9.5 (n = 4). These CCh and BK plateau levels values are significantly different (p < 0.006). When cells were rinsed with Ca\(^{2+}\)-free HBSS at the end of the experiments, the Ca\(^{2+}\) returned to the basal level (data not shown), indicating that Ca\(^{2+}\) entry from the extracellular space mediates the sustained elevation of Ca\(^{2+}\) under both CCh- and BK-stimulated conditions. These results suggested that CCh might be more effective than BK in stimulating Ca\(^{2+}\) entry via endogenous HEK-BKR21 Ca\(^{2+}\) channels.

To take a closer look at the comparative ability of CCh and BK to stimulate Ca\(^{2+}\) entry, we used another protocol to monitor the activation of Ca\(^{2+}\) entry pathways. In Fig. 2, we treated cells with either BK, CCh, or TG in a Ca\(^{2+}\)-free HBSS solution, and after internal pools were empty, we added HBSS containing Ca\(^{2+}\). The initial slope of the Ca\(^{2+}\) uptake curve was taken as a measure of the rate of Ca\(^{2+}\) accumulation. It is even more clear in this protocol that CCh stimulates Ca\(^{2+}\) uptake much better than does BK (CCh slope = 5.63 ± 0.51, n = 5, versus BK slope = 0.96 ± 0.11, n = 9, significantly different, p < 0.001). TG also was able to stimulate the uptake of Ca\(^{2+}\) much more effectively than BK (TG slope = 5.28 ± 0.65, n = 5; significantly higher than BK slope, p < 0.003). There was not a statistically significant difference between the Ca\(^{2+}\) uptake stimulated by CCh and TG.

Based on the observation that CCh and TG both stimulated Ca\(^{2+}\) uptake much more effectively than BK, it seemed that one possibility was that CCh and TG both gave a more complete depletion of internal Ca\(^{2+}\) stores, thereby producing more SOCE than seen with BK. Therefore, we next wanted to compare the ability of BK and CCh to deplete intracellular pools to determine whether CCh was simply more effective than BK at emptying the TG-sensitive Ca\(^{2+}\) pools. To test the relative ability of the two agonists to release stored Ca\(^{2+}\), we stimulated with one agonist in Ca\(^{2+}\)-free medium and then came back and stimulated with the other agonist and monitored how much additional Ca\(^{2+}\) could be released by the second agonist. The data in Fig. 3 indicate that there is considerable overlap between the BK-sensitive and CCh-sensitive Ca\(^{2+}\) pools. We see that CCh treatment releases most but not all of the BK-sensitive intracellular calcium pool. Likewise, BK treatment releases a significant portion of the CCh-sensitive intracellular calcium pool. In a set of three experiments, we observed that the area under the curve for the CCh response was 26,511 ± 1697 compared with 19,139 ± 2340 for BK, (significantly different, p < 0.05). It also appears that CCh is more effective than BK at releasing the Ca\(^{2+}\) pool sensitive to the other agonist. When CCh is the first agonist, the area under the BK curve is 15.4 ± 0.02% (n = 4) of the area under the first peak. When BK is the first agonist, the area under the CCh peak is 60.1 ± 0.01% (n = 3) of the area under the BK peak. These values are significantly different (p < 0.00002). We will return later to the question of whether this difference in Ca\(^{2+}\) release is of sufficient magnitude to explain the differential activation of Ca\(^{2+}\) entry. The data in Fig. 4 shows that the intracellular Ca\(^{2+}\) pool emptied by TG includes both the BK- and CCh-sensitive Ca\(^{2+}\) pools.
Differential Regulation of PMCA by CCh and BK

One consistent observation about the CCh-stimulated release of internal Ca\textsuperscript{2+} pools led us to examine the possibility that a subtle difference in the way CCh releases pool Ca\textsuperscript{2+} might provide an explanation for the difference in activation of Ca\textsuperscript{2+} entry between BK and CCh. We find that when we monitor the Ca\textsuperscript{2+} response to CCh in a Ca\textsuperscript{2+}-free medium and average that response over a large number of cells, we always see a double peak for the CCh response. Our initial question was whether this double peak was the result of two different populations of cells with slightly different time courses of Ca\textsuperscript{2+} release or the result of each individual cell responding with a double peak. Therefore, we monitored the release of Ca\textsuperscript{2+} in individual cells. In Fig. 5A, we see that individual HEK-BKR21 cells stimulated with 100 µM CCh show a double peak that is typical of the whole population of cells. This suggested that CCh might stimulate Ca\textsuperscript{2+} release from two separate intracellular Ca\textsuperscript{2+} pools with different kinetics of release and perhaps this could explain why CCh releases more Ca\textsuperscript{2+} than BK.

In an attempt to better resolve the two peaks of CCh-stimulated Ca\textsuperscript{2+} release, we went to a more rapid data acquisition protocol utilizing the non-ratio calcium dye Fluo-3. Without delays for filter switching, we obtained a more rapid data acquisition in individual cells, which enabled us to observe that the notched peak was not due to release from two separate Ca\textsuperscript{2+} pools but was more likely due to the repetitive release from the same pool. As seen in Fig. 5B, at faster rates of image capture, we could resolve a series of damped oscillations, which at slower rates of capture had fused into a declining plateau. One can see from the average response of 11 individual HEK-BKR21 cells in Fig. 5C that such a response averaged over 300–500 cells would mask the damped oscillations and give the dual peak seen earlier.

Because the comparison of Ca\textsuperscript{2+} pool depletion by BK and CCh suggested that a difference in pool depletion by CCh versus BK might explain the differences in Ca\textsuperscript{2+} uptake on the basis of differential activation of store-operated channels, we performed one more set of experiments to make sure the difference in Ca\textsuperscript{2+} uptake was due to increased channel activity and not due to a change in Ca\textsuperscript{2+} pump activity. Although an initial ion uptake rate such as we measured in Fig. 2 is normally considered to be independent of ion efflux, this may not be the case for Ca\textsuperscript{2+}. First, the cytosolic Ca\textsuperscript{2+} concentration when external Ca\textsuperscript{2+} is added is not very different from the steady state basal Ca\textsuperscript{2+} concentration for cells in HBSS, where Ca\textsuperscript{2+} influx is equal to Ca\textsuperscript{2+} efflux. Second, it is likely that the Ca\textsuperscript{2+} concentration near the membrane rises much more rapidly than in the general cytosol and that the PMCA may kick in much steeper dependence than predicted from pump kinetics

![Fig. 4. TG depletes both the BK- and CCh-responsive intracellular Ca\textsuperscript{2+} pools.](image)

![Fig. 5. CCh induced Ca\textsuperscript{2+} release from individual HEK-BKR21 cells.](image)
alone. Thus, we may be measuring a combination of influx and 
Ca\(^{2+}\) pump efflux very early in the Ca\(^{2+}\) uptake curve in Fig. 2. 
Thus, we went back to the type of experiment seen in Fig. 2, but 
instead of monitoring Ca\(^{2+}\) entry, we chose to monitor Ba\(^{2+}\) entry, since it is known that Ba\(^{2+}\) is not pumped by Ca\(^{2+}\) -
ATPases either into internal stores or out of the cell (16, 17).
The data in Fig. 6 show that when we use Ba\(^{2+}\) uptake to 
monitor channel activity, we observed a dramatically different 
order of efficacy. Although CCh was still the most effective 
agonist, BK was now more effective than TG at stimulating 
Ba\(^{2+}\) entry (CCh slope 0.0038 ± 0.0001, n = 6; BK slope = 0.00265 ± 0.0001, n = 12; TG slope = 0.00147 ± 0.00003, n = 6). These values are statistically different, p < 0.0001. Note 
that the scale of these slopes is different from those reported 
earlier because we are plotting 340/380 ratios for Ba\(^{2+}\) experi-
ments and Ca\(^{2+}\) values for Ca\(^{2+}\) experiments. At this point, 
the data suggest that, for some reason, BK stimulates Ca\(^{2+}\) uptake much less effectively than CCh, which contrasts with 
the relatively minor differences in their effect on Ba\(^{2+}\) uptake. 
This could indicate either a differential permeability of Ca\(^{2+}\) versus Ba\(^{2+}\) through channels activated by store depletion, or 
by CCh and BK, or it could indicate that BK stimulates re-
moval of cytosolic Ca\(^{2+}\) via Ca\(^{2+}\)-ATPases. We will return to 
this issue later in the results section.
When we were performing the experiments in Fig. 1, to 
examine the height of the plateau phase of Ca\(^{2+}\) in response to 
CCh, we noted one very interesting phenomenon that sug-
gested that CCh also may modify the Ca\(^{2+}\) pump activity. The data in Fig. 7 show a comparison of the rates of decline of 
cytosolic Ca\(^{2+}\) after removal of Ca\(^{2+}\) either in the continued 
presence of CCh or in the absence of CCh. The data in Fig. 7 
show that removal of Ca\(^{2+}\) in the absence of CCh results in a 
much steeper decline of cytosolic levels than seen in the pres-
ence of CCh. The data in Fig. 7A show that the order in which 
the media changes are performed is not important. The return 
to basal after the removal of external Ca\(^{2+}\) is always faster in 
the absence of CCh. The rate constant for the first-order decline 
in 0 Ca\(^{2+}\) medium was 0.094 ± 0.007, which was significantly 
different (p < 0.00002, n = 9) from the rate constant for the 
decline in 0 Ca\(^{2+}\)/CCh, which was 0.039 ± 0.003 (n = 9). To rule 
out the possibility that the Na\(^+\)/Ca\(^{2+}\) exchanger could play an 
important role in the Ca\(^{2+}\) efflux process, we performed exper-
iments similar to the ones in Fig. 7, except in the absence of 
external Na\(^+\). We found that CCh still modified the rate con-
stant for Ca\(^{2+}\) removal even when n-methyl-d-glucamine was 
substituted for the external Na\(^+\) ions. The rate constants for 
Ca\(^{2+}\) decline were 0.147 ± 0.015 (n = 6) in 0 Ca\(^{2+}\), Na\(^+\) -free 
compared with 0.074 ± 0.006 (n = 6) in CCh/0 Ca\(^{2+}\)/Na\(^+\)-free 
(significantly different, p < 0.001). Although both the rate 
constants were higher than those observed in Na\(^+\)-containing
medium, there was still a 2-fold difference in rate constants in response to CCh.

A trivial explanation for the slow return to basal levels in the presence of CCh could be that, in Ca\(^{2+}\)-free medium, the Ca\(^{2+}\) is pumped both out of the cell and into the ER, whereas in the presence of CCh the impact of the ER pump on the removal of Ca\(^{2+}\) from the cytosol is reduced due to the continual loss of Ca\(^{2+}\) from the ER via the inositol 1,4,5-trisphosphate receptor.

To test this possibility, we performed a similar experiment where the pump capacity of the ER is compromised by the presence of TG. A similar approach to quantifying the Ca\(^{2+}\) efflux rate in platelets has been reported previously (18). In the initial part of Fig. 8, we see the previously described slower decline of Ca\(^{2+}\) in the presence of CCh, as compared with the very rapid decline in Ca\(^{2+}\)-free medium in the absence of CCh. We then added TG to inhibit pumping into the ER store, resulting in store depletion as well as establishment of a plateau level due to activation of the SOCE pathway. Now if the sole effect of CCh on Ca\(^{2+}\) removal were to prevent accumulation of Ca\(^{2+}\) into the ER, we should see the same rate of decline of Ca\(^{2+}\) to basal levels in Ca\(^{2+}\)-free medium containing TG as we saw in the CCh + Ca\(^{2+}\)-free medium earlier in the experiment (rate constant was 0.118 \pm 0.004, n = 3; significantly different from the TG condition, \(p < 0.001\)). It was nowhere near as slow as the decline seen in the Ca\(^{2+}\)-free medium containing CCh (rate constant was 0.0391 \pm 0.005, n = 3; significantly different from the TG condition, \(p < 0.01\)). This suggests that a large portion of the reduction of Ca\(^{2+}\) pump activity induced by CCh may be due to inhibition of the plasma membrane Ca\(^{2+}\) pump.

Earlier under “Results” we mentioned that perhaps BK might be stimulating plasma membrane pump activity. The initial data supporting this hypothesis are shown in Fig. 9. In Fig. 9A, the data indicate that there is a dramatic difference in the levels of Ca\(^{2+}\) uptake stimulated by low versus high doses of BK. Although stimulation of cells with 1 nM BK releases much less intracellular Ca\(^{2+}\) than stimulation with 100 nM BK, the initial Ca\(^{2+}\) uptake stimulated by 1 nM BK is much higher (~6.6-fold) than that stimulated by 100 nM BK (the slope for 1 nM BK = 6.32 \pm 1.6, n = 7; the slope for 100 nM BK = 0.96 \pm 0.11, n = 9; statistically different, \(p < 0.01\)). However, if we measure Ba\(^{2+}\) entry in response to low and high doses of BK we see just the opposite. The high dose of BK stimulates both a higher level of Ca\(^{2+}\) release and a slightly higher level of Ba\(^{2+}\) entry (~6.3-fold) than the slope for BK (slope for 1 nM BK = 6.32 \pm 1.6, n = 7; the slope for 100 nM BK = 0.96 \pm 0.11, n = 9; statistically different, \(p < 0.01\)). However, if measure Ba\(^{2+}\) entry in response to low and high doses of BK we see just the opposite. The high dose of BK stimulates both a higher level of Ca\(^{2+}\) release and a slightly higher level of Ba\(^{2+}\) entry (~6.3-fold) than the slope for BK (slope for 1 nM BK = 6.32 \pm 1.6, n = 7; the slope for 100 nM BK = 0.96 \pm 0.11, n = 9; statistically different, \(p < 0.01\)). However, if we measure Ba\(^{2+}\) entry in response to low and high doses of BK we see just the opposite. The high dose of BK stimulates both a higher level of Ca\(^{2+}\) release and a slightly higher level of Ba\(^{2+}\) entry (~6.3-fold) than the slope for BK (slope for 1 nM BK = 6.32 \pm 1.6, n = 7; the slope for 100 nM BK = 0.96 \pm 0.11, n = 9; statistically different, \(p < 0.01\)). However, if we measure Ba\(^{2+}\) entry in response to low and high doses of BK we see just the opposite. The high dose of BK stimulates both a higher level of Ca\(^{2+}\) release and a slightly higher level of Ba\(^{2+}\) entry (~6.3-fold) than the slope for BK (slope for 1 nM BK = 6.32 \pm 1.6, n = 7; the slope for 100 nM BK = 0.96 \pm 0.11, n = 9; statistically different, \(p < 0.01\)). However, if we measure Ba\(^{2+}\) entry in response to low and high doses of BK we see just the opposite. The high dose of BK stimulates both a higher level of Ca\(^{2+}\) release and a slightly higher level of Ba\(^{2+}\) entry (~6.3-fold) than the slope for BK (slope for 1 nM BK = 6.32 \pm 1.6, n = 7; the slope for 100 nM BK = 0.96 \pm 0.11, n = 9; statistically different, \(p < 0.01\)). However, if...
UV light results in a substantial peak of Ca$^{2+}$ that rapidly declines with time when the train of pulses is stopped. The magnitude of the peak is reproducible as seen in Fig. 10A. However, if cells are stimulated with either CCh or BK in between light pulses, then the peak heights from the UV-induced release of Ca$^{2+}$ are dramatically different. Stimulation of the cells with CCh in between the two peaks results in a peak area for the second peak that is significantly higher than the one seen before CCh stimulation (Fig. 10C). This suggests that CCh slows the removal of Ca$^{2+}$ from the cytosol and, therefore, must inhibit some pump activity. In contrast to CCh, the effect of BK is to stimulate the pump. The addition of 100 nM BK between the two light pulses dramatically reduces the Ca$^{2+}$ peak area in response to the second pulse (Fig. 10B). This suggests that BK increases the rate of removal of Ca$^{2+}$ from the cytosol. A statistical analysis of the ratio of the area under the curves for the first peak and second peak is shown in Fig. 10D. Based on 15 separate experiments CCh produces a second peak that is $2.05 \pm 0.24$ ($n = 15$) times larger than the first peak (significantly different than a ratio of 1, $p < 0.003$), whereas BK results in a second peak that is $0.61 \pm 0.12$ times as large as the first peak (significantly different from 1, $p < 0.004$).

Because the results of Fig. 8 indicate that CCh must have an effect beyond reducing Ca$^{2+}$ removal via the ER pump, we chose to focus further investigations on the PMCA. Previously, work from Vanaman and coworkers (19) indicates that tyrosine phosphorylation of PMCA can significantly inhibit its pump activity. Thus, we investigated whether the levels of tyrosine phosphorylation of PMCA changed in response to CCh and BK.

The data in Fig. 11A are from a representative experiment that shows a substantial basal level of tyrosine phosphorylation of PMCA. The PMCA tyrosine phosphorylation level is greatly enhanced by the addition of CCh and significantly reduced by the addition of BK. The data in Fig. 11B show the statistics from a total of seven phosphorylation experiments. Because of the substantial variation in the amount of basal phosphorylation from experiment to experiment, we expressed the data in Fig. 11B as the ratio of the stimulated tyrosine phosphorylation level to the basal tyrosine phosphorylation level for each individual experiment. We found that CCh greatly increased the level of tyrosine phosphorylation of PMCA, with the mean fold increase over basal levels being 8.8 $\pm$ 3.1 (this value is significantly higher than a ratio of 1, predicted for no stimulation, $p < 0.015$). In contrast, BK reduced the level of tyrosine phosphorylation of PMCA to a basal level of 0.23 $\pm$ 0.09 (this value is significantly lower than a ratio of 1 predicted for no inhibition, $p < 0.00002$). Thus, the differential response of tyrosine phosphorylation of PMCA fits well with the differential Ca$^{2+}$ pump activity monitored in Figs. 7 and 10.

**DISCUSSION**

It has been recognized for some time that GPCRs can couple to a variety of different G proteins and thereby initiate a variety of signaling pathways. Thus, some GPCRs will couple to G$q$ or G$i$ and have opposing effects on cAMP production, whereas others will couple to G$q$ and initiate signaling via the phospholipase C pathway. Some receptors may even couple to a variety of different G proteins and thereby initiate a variety of signaling pathways. Thus, some GPCRs will couple to G$q$ and initiate signaling via the phospholipase C pathway. Some receptors may even couple to G$q$ and initiate signaling via the phospholipase C pathway.

It should therefore stimulate similar pathways. A direct comparison to our early observations that TG was more effective in stimulating Ca$^{2+}$ entry than BK. This was rather puzzling since both BK and CCh are known to couple to G$q$ and should therefore stimulate similar pathways. A direct comparison showed that CCh stimulated a higher Ca$^{2+}$ plateau level than BK in HEK-BKR21 cells (Fig. 1) and after depletion of Ca$^{2+}$ in a Ca$^{2+}$-free medium CCh was much more effective in...
stimulating Ca$^{2+}$ entry than was BK. However, in contrast to studies overexpressing hTrpC3 in HEK-293 cells, CCh was not significantly better than TG at stimulating Ca$^{2+}$ entry via endogenous channels in our HEK-BKR21 cells (Fig. 2).

The initial hypothesis tested was that CCh was more effective at emptying internal Ca$^{2+}$ stores and, thereby, more effective at stimulating SOCE, and studies to investigate the level of Ca$^{2+}$ release in Ca$^{2+}$-free medium soon indicated that CCh is better at releasing Ca$^{2+}$ stores (Fig. 3). Although both BK and CCh released significant amounts of the internal Ca$^{2+}$ stores, the addition of TG after either BK or CCh stimulation in Ca$^{2+}$-free medium resulted in a substantial release of internal Ca$^{2+}$ (data not shown). On the other hand, TG could fully deplete both the BK- and CCh-sensitive Ca$^{2+}$ pools. Thus, if we were looking solely at SOCE, one would predict that TG might be much more effective at stimulating Ca$^{2+}$ entry than CCh as well as being more effective than BK. Thus, the differential activation of SOCE by CCh and BK does not appear to be sufficient to explain the large difference in activation of Ca$^{2+}$ entry. There are, however, several problems with this argument. First, results in other cell types suggest that TG depletes Ca$^{2+}$ stores in addition to those residing in the inositol 1,4,5-trisphosphate-sensitive store (20), which is presumably the one coupled to SOCE. Second, activating these GPCRs could activate receptor-stimulated Ca$^{2+}$ entry pathways in addition to store-operated channels.

We next investigated whether CCh might release Ca$^{2+}$ from a pool in addition to the Ca$^{2+}$ pool that was accessed by BK. This possibility was suggested by the double peak observed when CCh is added to cells in a Ca$^{2+}$-free medium (Fig. 5A). Perhaps the two peaks resulted from release from two kinetically distinct pools. More rapid data acquisition using a non-ratio Ca$^{2+}$ indicator demonstrated that the double peak we had previously seen was simply the result of averaging between cells whose individual response showed damped oscillations (Fig. 5, B and C). Thus, rather than indicating release from two separate pools, the double peak was more likely to be the result of “re-release” from the same pool.

Before launching an intense investigation of whether multiple Ca$^{2+}$ entry pathways were stimulated by CCh but not by BK, we first investigated whether some differences in pump activity might explain the higher CCh- than BK-stimulated Ca$^{2+}$ entry. To begin, we examined whether the differential effects of CCh and BK on Ca$^{2+}$ entry could also be observed when Ba$^{2+}$ is used to trace channel activity. Although Ba$^{2+}$ passes through many Ca$^{2+}$ channels, it is not pumped by Ca$^{2+}$-ATPases, thereby allowing us to separate effects on Ca$^{2+}$ pump activity from the regulation of channel activity. We observed that with Ba$^{2+}$ as a tracer, the CCh-stimulated entry was only 1.4 times the level of BK-stimulated entry (Fig. 6). This was in dramatic contrast to the Ca$^{2+}$ results, where the CCh-stimulated entry was 5.5 times the BK-stimulated entry. Both the BK-stimulated and the CCh-stimulated Ba$^{2+}$ entry were higher than the TG-stimulated Ba$^{2+}$ entry, indicating that both CCh and BK stimulate entry pathways in addition to the TG-stimulated entry pathway. Furthermore, the large difference in CCh- and BK-stimulated Ca$^{2+}$ uptake must be due to effects on Ca$^{2+}$ pumping rather than Ca$^{2+}$ entry via channels;
that is, either CCh must inhibit Ca\(^{2+}\)/H\(11001\) pump activity or BK must stimulate Ca\(^{2+}\)/H\(11001\) pump activity.

When we were comparing the effects of BK and CCh on Ca\(^{2+}\) plateaus in the Ca\(^{2+}\)/H\(11001\)-containing medium, we observed another phenomenon that seemed to support the hypothesis that CCh has an inhibitory effect on Ca\(^{2+}\)/H\(11001\) pump activity. We found that different results were obtained when Ca\(^{2+}\)/H\(11001\) was removed in the continued presence of CCh versus when Ca\(^{2+}\)/H\(11001\) was removed in the absence of CCh. When we switched the medium to Ca\(^{2+}\)-free in the absence of CCh we observed a very sharp decline of [Ca\(^{2+}\)/H\(11001\)]. This was in contrast to the very slow decline observed when TG was added to inhibit the sarcoplasmic reticulum calcium ATPase pump, the rate of decline was still much more rapid than that observed in the presence of CCh. This suggested that a significant portion of CCh effect is likely to be on the PMCA rather than on the sarcoplasmic reticulum calcium ATPase.

Although the data appear to support an inhibitory effect of CCh on Ca\(^{2+}\) pump activity, the evidence points to BK having a stimulatory effect on Ca\(^{2+}\) pump activity. Because BK can stimulate the release of a large fraction of the TG-sensitive Ca\(^{2+}\) store one would expect that the effect of BK on Ca\(^{2+}\) entry would be close to that seen with TG. However, instead we see that 100 nM BK stimulates only 18% of the Ca\(^{2+}\) uptake stimulated by TG (Fig. 2). This was the first indication that BK might stimulate Ca\(^{2+}\) pump activity. This hypothesis was further supported by the observation that BK actually stimulated a level of Ba\(^{2+}\) entry that was 1.8 times that seen with TG (Fig. 6), arguing that the lower BK effect on Ca\(^{2+}\) entry must be via a Ca\(^{2+}\) pump effect. In addition, the effect of BK on Ca\(^{2+}\) pump activity seemed to be more visible at higher BK doses. The initial Ca\(^{2+}\) entry was 6.5-fold higher at 1 nM BK than at 100 nM BK despite the fact that the higher dose of BK released more Ca\(^{2+}\) from internal stores (Fig. 9). In contrast, the Ba\(^{2+}\) entry was 1.3-fold higher at 100 nM BK than at 1 nM BK. These data indicate that one effect of BK on Ca\(^{2+}\) uptake results from a stimulation of Ca\(^{2+}\) pump activity at higher BK doses.

The hypothesized effect of BK and CCh on Ca\(^{2+}\) pump activity was further investigated by producing test pulses of Ca\(^{2+}\) in the cytosol and monitoring the way this Ca\(^{2+}\) was handled in

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**Fig. 10. Effect of CCh or BK on the peak height of [Ca\(^{2+}\)/H\(11001\)] after flash photolysis of NP-EGTA-AM.** HEK-BKR21 cells grown on coverslips were preincubated with 6 \(\mu\)M caged calcium compound NP-EGTA-AM before loading with fluorescence indicator Fluo-3-AM. UV exposure was utilized to release Ca\(^{2+}\) from the caged calcium compound. A, Ca\(^{2+}\) response to repeated, brief UV exposures. B, Ca\(^{2+}\) response to repeated, brief UV exposures before and after CCh (100 \(\mu\)M) stimulation. C, Ca\(^{2+}\) response to repeated, brief UV exposures before and after BK (100 nM) stimulation. D, the statistical analysis of 15 separate experiments comparing the ratio of the area under the second peak to the area under the first 1.
we chose the area under the curve as the best measure of the effect of CCh and BK in these experiments.

Because the data in Na\(^+\)-free medium ruled out participation of the Na\(^+\)/Ca\(^{2+}\) exchanger and the data in Fig. 8 suggested that a significant portion of the CCh effect was not mediated by the sarcoendoplasmic reticulum calcium ATPase pumps, we began to consider how BK and CCh might differentially affect the PMCA. Because a previous publication reports that the tyrosine phosphorylation of PMCA led to the inhibition of its pump activity (19), we investigated the effects of BK and CCh on the tyrosine phosphorylation of PMCA. We consistently observed a dramatic increase (~8-fold) in tyrosine phosphorylation of the PMCA in response to the addition of CCh (Fig. 11). Based on results from Vanaman and co-workers (19), the increased PMCA tyrosine phosphorylation would be consistent with an inhibitory effect of CCh on the PMCA. In contrast, we consistently observed a decrease in tyrosine phosphorylation (to ~20% of control level) after stimulation of cells by BK (Fig. 11), an observation that would be consistent with a stimulation of PMCA activity.

In summary, we have demonstrated that two compounds which are agonists for GPCRs coupled to G\(_q\) have opposite effects on PMCA activity, and these effects appear to be mediated via a differential tyrosine phosphorylation of PMCA. These observations clearly point out the fact that we cannot treat all agonists of GPCR linked to G\(_q\) as if they will all regulate cytosolic Ca\(^{2+}\) levels in a similar manner. There clearly are subtle differences in the way they regulate [Ca\(^{2+}\)], and it is clear that we must learn more about these subtle differences and the mechanisms for such differential regulation before we will fully understand the regulation of cytosolic Ca\(^{2+}\) levels. Studies are under way to investigate the tyrosine kinase involved in the tyrosine phosphorylation of PMCA and the mechanism by which they are regulated.

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